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(54) Title: METHODS OF INDUCING IMMUNITY TO LYME DISEASE AND A COLORIMETRIC ASSAY FOR BORRELIACIDAL ACTIVITY OF ANTISERA

(57) Abstract

A simple, colorimetric borreliacidal assay for the determination of borreliacidal activity of immune serum to *Borrelia burgdorferi* is disclosed. Also disclosed is a vaccine for *Borrelia burgdorferi* as well as methods of inducing immunity to bacteria causing Lyme disease, including *B. burgdorferi* and *B. garinii* by administering the vaccines of the present invention. Also disclosed are kits comprising one or more vials comprising the elements of the *Borrelia burgdorferi* vaccine. Also disclosed are vaccine formulations for Lyme disease.

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METHODS OF INDUCING IMMUNITY TO LYME DISEASE AND A COLORIMETRIC ASSAY FOR BORRELIACIDAL ACTIVITY OF ANTISERA

This is a continuation-in-part (CIP) application of the copending U.S. Patent Application, Serial No. 08/025,379, filed February 26, 1993, the entire disclosure of which is hereby incorporated by reference herein. Applicants claim benefit to the earlier priority date of said U.S. Application, Serial No. 08/025,379, filed February 26, 1993.

Field of the Invention

The present invention is in the field of medicinal chemistry. In particular, the invention is related to vaccines for *Borrelia burgdorferi* comprising a saponin, and the use thereof to immunize animals.

Background of the Invention

Lyme disease is a tick-borne multisystemic disorder characterized by early erythema chronicum migrans, late arthritis, and cardiac and neurologic manifestations in humans and animals (Steere, A.C., *N. Engl. J. Med. 321*:586-596 (1989); Kornblatt *et al., J. Am. Vet. Med. Assoc. 186*:960-4 (1985); Lissman *et al., J. Am Vet. Assoc. 185*:219-20 (1984)). Caused by the spirochete *Borrelia burgdorferi* (Burgdorfer, W., *et al., Science 261*:1317-1319 (1982)), Lyme disease is the most common tick-borne zoonosis occurring in humans and dogs (Steere, A.C., *N. Engl. J. Med. 321*:586-96 (1989)). Morbidity of Lyme disease can be as high as 9% in highly endemic area (Alpert *et al., NY State J. Med. 92*:5-8 (1992)). Thus far, at least three genospecies of Lyme disease spirochetes have been recognized based on genetic and molecular determinants (Baranton *et al., Int. J. Sys. Bacteriol. 42*:378-83 (1992)). Most North American isolates and partial European isolates are *Borrelia burgdorferi sensu stricto*. Many European and Asian

isolates belong to *Borrelia garinii sp. nov.*, and group VS461 (Baranton *et al.*, *Int. J. Sys. Bacteriol. 42*:378-83 (1992); Park *et al.*, *J. Clin. Microbiol. 31*:1831-7 (1993)).

Humoral immunity is a major protective mechanism against this bacterial infection or disease (Burgdorfer, W., *et al.*, *Science 261*:1317-1319 (1982); Simon, M.M., *et al.*, *Immunol. Today 12*:11-16 (1991); Schaible, U.E., *et al.*, *Proc. Natl. Acad. Sci. USA 8*:3768-3772 (1990); Fikrig, E., *et al.*, *Science 250*:553-556 (1990)). Convalescent human sera are able to kill the spirochete in the presence of complement *in vitro*, possibly by altering the bacterial outer membrane to allow the formation of an effective membrane attack complex (Kochi and Johnson, *Infect. Immun. 56*:314-321 (1988); Kochi, S.K., *et al.*, *J. Immunol. 146*:3964-3970 (1991)). Heat-inactivated rat antisera to *B. burgdorferi* or mouse monoclonal antibody to a surface epitope of the spirochete in the absence of complement are also capable of lysing this spirochete (Pavia, C.S., *et al.*, *J. Infect. Dis. 163*:656-659 (1991); Coleman and Benach, "Characterization of antigenic determinants of *Borrelia burgdorferi* shared by other bacteria," *J. Infect. Dis. 165*:658-666 (1992)). Furthermore, *in vitro* borreliacidal activity of hamster immune sera to *B. burgdorferi* with complement is reported to correlate with *in vivo* protection (Lovrich, S.D., *et al.*, *Infect. Immun. 59*:2522-2528 (1991)).

Recent studies on Lyme disease have contributed to the better understanding of the pathogenesis of Lyme disease and host immune responses to *B. burgdorferi* (Garcia-Monco *et al.*, *Rheum. Clin. N. Am. 15*:711-26 (1989); Szczepanski *et al.*, *Microbiol. Rev. 55*:21-34 (1991)). Borreliacidal and antiborrelial activity of specific antibody, opsonophagocytosis and subsequent killing constitute the important protective mechanisms against borrelial infection (Benach *et al.*, *J. Infect. Dis. 150*:497-507 (1984); Kochi *et al.*, *Infect. Immun. 56*:314-21 (1988); Pavia *et al.*, *J. Infect. Dis. 163*:656-9 (1991); Ma *et al.*, *J. Microbiol. Methods 17*:145-53 (1993); Peterson *et al.*, *Infect. Immun. 46*:608-11 (1984); Schaible *et al.*, *Proc. Natl. Acad. Sci. USA 8*:3768-72 (1990)). Immunoprotection studies in mice have shown that the

outer surface proteins A (OspA) and B (OspB) of *B. burgdorferi* are protective immunogens and are the candidates for vaccine development (Schaible *et al.*, *Proc. Natl. Acad. Sci. USA* 8:3768-72 (1990); Fikrig *et al.*, *Science 250*:553-6 (1990); Fikrig *et al.*, *Infect. Immun. 60*:657-661 (1992)). These early observations indicate that protection was possible against *B. burgdorferi* but not against other species of *Borrelia*.

Canine immune responses to the infection of Lyme disease spirochetes is poorly understood. Low and high antibody responses have been detected in the spirochete-positive and asymptomatic dogs (Kornblatt *et al.*, *J. Am. Vet. Med. Assoc. 186*:960-4 (1985); Lissman *et al.*, *J. Am. Vet. Assoc. 185*:219-20 (1984); Burgess, E.C., *Lab. Ani. Sci. 36*:288-90 (1986)). Antibody response to OspA were rarely detectable in naturally exposed dogs. (Greene *et al.*, *J. Clin. Microbiol. 26*:648-53 (1988)). This observation is similar to the serological findings in human Lyme disease patients that antibodies to OspA and OspB are rarely detectable even at late stage of Lyme disease (Craft *et al.*, *J. Clin. Invest. 78*:934-39 (1986); Coleman *et al.*, *J. Infect. Dis. 155*:756-65 (1987)). Thus, whether or not OspA and OspB based vaccine would stimulate protective immune responses in Lyme disease hosts needed to be addressed.

Borreliacidal activity of immune sera is usually determined by an *in vitro* assay adapted from an antileptospiral assay (Kochi and Johnson, *Infect. Immun. 56*:314-321 (1988)). Because *B. burgdorferi* does not reliably form isolated colonies on currently available agar medium, spirochete killing by the bactericidal antibody is determined by dark-field microscopy on the basis of loss of motility, refractility, or extensive surface blebbing (Kochi and Johnson, *Infect. Immun. 56*:314-321 (1988); Kochi, S.K., *et al.*, *J. Immunol. 146*:3964-3970 (1991); Lovrich, S.D., *et al.*, *Infect. Immun. 59*:2522-2528 (1991); Callister, S.M., *et al.*, *J. Clin. Microbiol. 29*:1773-1776 (1991)). Enumeration of live spirochetes, however, is difficult since antibody specific to the outer surface proteins or lysate of *B. burgdorferi* strongly agglutinates the spirochetes (Coleman and Benach, "Characterization of antigenic

determinants of *Borrelia burgdorferi* shared by other bacteria," *J. Infect. Dis.* *165*:658-666 (1992); Ma, J., *et al.*, "Antibody mediated microagglutination of *Borrelia burgdorferi* correlates with antibody titers to rec OspA," *V Int. Conf. Lyme Borreliosis*, Arlington, VA, USA (1992)). This agglutination also makes it difficult to examine the viability of the agglutinated bacteria. To confirm the observation obtained by dark-field microscopy, a radioactive incorporation assay or spirochete culture is conducted as well (Kochi and Johnson, *Infect. Immun.* *56*:314-321 (1988); Pavia, C.S., *et al.*, *J. Infect. Dis.* *163*:656-659 (1991); Lovrich, S.D., *et al.*, *Infect. Immun.* *59*:2522-2528 (1991); Callister, S.M., *et al.*, *J. Clin. Microbiol.* *29*:1773-1776 (1991)). The current borreliacidal assays, therefore, are tedious and time-consuming, and lack reliability. There is also a safety issue surrounding the use of radioactive materials and observing the pathogenic spirochete under a dark-field microscope.

Summary of the Invention

The present invention is directed to a new colorimetric borreliacidal assay (CBA) for determination of the bactericidal activity of antiserum to *B. burgdorferi*, which is simple, reliable, and exhibits an easily measurable end-point. The results of the CBA correlated well with those of both direct dark-field microscopy and [^{3}H]-thymidine incorporation assay (TIA). This colorimetric microtiter assay was measured by a microplate reader and thus the results can be processed automatically using an appropriate computer program.

In particular, the invention relates to a method for the determination of the bactericidal activity of an antiserum to *B. burgdorferi*, which comprises

(a) contacting said antiserum with a sample containing *B. burgdorferi* and a suitable color pH indicator such as phenol red, for measurement of bacterial growth; and

(b) measuring the absorbance of the sample;

wherein high absorbance, when compared to a control sample which does not contain *B. burgdorferi*, is an indication of high bactericidal activity.

Color pH indicators are known in the art and may be readily obtained from the various suppliers. For example, various color pH indicators are described in the 1993 catalogue of Sigma Chemical Co., St. Louis, Missouri, at page 1438.

The invention also relates to a vaccine, comprising OspA, OspB or fragments thereof; and a saponin adjuvant.

More specifically, the invention relates to a vaccine, comprising full-length lipoproteins OspA and/or OspB; and a saponin adjuvant such as QS-21.

The invention also relates to a method of inducing immunity to bacteria causing lyme disease, e.g., *B. burgdorferi* and *B. garinii*, in an animal, comprising administering to the animal a vaccine comprising OspA, OspB or fragments thereof; and a saponin adjuvant.

More specifically, the invention relates to a method of inducing immunity to *B. burgdorferi* and other species of the genus *Borrelia* in an animal, comprising administering to the animal a vaccine comprising full-length lipoproteins OspA and/or OspB; and a saponin adjuvant such as QS-21.

An advantage of the present invention is that the immunogenicity of OspA- and OspB-based subunit vaccine is greatly enhanced by using the full-length lipoproteins OspA and OspB.

A further advantage of the present invention is that the immunogenicity of Osp subunit vaccine is further enhanced by the adjuvant QS-21.

Another advantage of the present invention is that a preferred vaccine formulation comprising OspA, OspB, and QS-21 displays borrelicidal activity against not only the homologous and closely related strains, but also against the heterologous and different genospecies of lyme disease spirochetes as well as other species of the genus *Borrelia*.

Moreover, the OspA- and OspB-based vaccine of the present invention elicits synergistically higher functional humoral immune response than a single protein-based vaccine.

Description of the Figures

Figure 1 depicts the colorimetric borreliacidal assay of mouse antisera to *B. burgdorferi* strain B31 against the homologous strain. Borrelial culture at logarithmic growth phase were centrifuged for 8 min. at 9000 x g at 15°C and resuspended in fresh mBSK. Ninety five μl of the borrial suspension (containing approximately 4 x 10^6 of spirochetes) and 5 μl of guinea pig compiement were mixed with an equal volume of the serially diluted (column 1 to 12) heat-inactivated mouse antisera (row A to C), normal sera (row D to F), and mBSK control row (row G to H) in mBSK containing 120 μg of phenol red in a microtiter plate, and incubated for 48 h at 32°C. Red color (dark) indicates borrelial death; yellow color (light) represents borrelial survival and growth.

Figure 2 depicts a graph showing the correlation of the colorimetric assay with the [^{3}H]thymidine incorporation assay. One hundred μl of *B. burgdorferi* strain B31 (containing about 8 x 10^6 spirochetes) was serially diluted in mBSK in 96-well plates, and incubated with an equal volume of mBSK-PR for 30 h at 32°C. Twenty μl of [^{3}H]thymidine in mBSK (2 μCi) were added to each well and the plates were incubated for another 18 h for pulse-labelling the live spirochete (Pavia, C.S. *et al., J. Infect. Dis 163*:656-659 (1991)). The absorbance at 562/630 nm (open circles) was measured by a microplate reader, and the radioactivity (closed circles) measured as counts per minutes by a scintillation counter. As shown in the figure, the results of CBA correlated with those of TIA (R = 0.977). The error bars represent the standard deviation of 3 measurements.

Figure 3 depicts a graph showing the changes of absorbance at 562/630 nm of the colorimetric borreliacidal assay with mouse antisera to OspA-B31 against the homologous strain. The assay was done as described in the description of Figure 1. The plate was incubated at 32°C for 120 h, and the absorbance measured using a microtiter reader before and every 24 h after

incubation. Each serum dilution was performed in triplicate. The error bars represent the standard deviation of 3 measurements.

Figure 4 depicts a graph showing the significant inhibition of the decrease in absorbance at 562/630 nm of the colorimetric borreliacidal assay by mouse antisera to *B. burgdorferi* stain B31. The serially diluted, heat-inactivated antisera or normal sera were incubated with the strain B31 in the presence of complement as described above in the description of Figure 1. Each serum dilution was performed in triplicate. The absorbance was measured after 48 h incubation. High absorbance indicates borrelial death; the low absorbance represents borrelial survival and growth. The error bars represent the standard deviation of 3 measurements.

Figure 5 depicts a graph showing the average borreliacidal activity of canine antisera to truncated OspA and/or OspB derived from *B. burgdorferi* strain B31 against the homologous strain.

Figure 6 depicts a graph showing the average borreliacidal activity of canine antisera to truncated OspA and/or OspB derived from *B. burgdorferi* strain B31 against the heterologous California strain CA-2-87.

Figure 7 depicts a graph showing the borreliacidal activity of canine antisera to truncated OspA and/or OspB derived from *B. burgdorferi* strain B31 against the homologous strain.

Figure 8 depicts a graph showing the borreliacidal activity of canine antisera to truncated OspA and/or OspB derived from *B. burgdorferi* strain B31 against the heterologous California strain CA-2-87.

Figure 9 depicts a graph showing the borreliacidal activity of C3H/Hej female mice that had been immunized twice with either 25 μg of truncated OspA, 25 μg of truncated OspB, or 25 μg of truncated OspA and 25 μg of truncated OspB.

Figure 10 depicts a graph showing the borreliacidal activity of C3H/Hej female mice that had been immunized twice with either 25 μg of truncated OspA + 20 μg of QS21, 25 μg of truncated OspB + 20 μg of

QS21, or 25 μ of truncated OspA and 25 μg of truncated OspB + 20 μg of QS21.

Figure 11 depicts a fluorograph of nitrocellulose membrane showing radiolabeling of FLOspA and FLOspB by [9,10-^{3}H]palmitic acid. *Escherichia coli* strain MZ-1 harboring *ospA* or *ospB* gene was grown in LB broth to log phase at 32°C. [9,10-^{3}H]palmitic acid was added to the culture and incubated for 2 h at 42°C. Bacteria were lysed, and OspA and OspB precipitated using specific MAbs described in the specification, by modification of the procedure (Katona *et al., Infect. Immun. 60*:4995-5003 (1992)). Samples were boiled for 5 min, subjected to SDS-PAGE, and transferred to nitrocellular membrane. The membrane was then treated with En^{3}Hance spray (Dupont-NEN, Boston, MA) and exposed to film. 1, FLOspA; 2, TOspA; 3, FLOspB; 4, TOspB. Molecular weight markers in KDa.

Figure 12 depicts antibody isotype titers of canine antisera to various experimental vaccines. Beagles at age of 12 and 16 weeks were immunized subcutaneously twice with various experimental vaccines. Immune sera were isolated two weeks after last immunization, and assayed by ELISA using plates coated with *B. burgdorferi* antigens (Cambridge Biotech Corporation, Worcester, MA; Lindenmayer *et al., J. Clin. Microbiol. 28*:92-6 (1990)). Antibody isotypes were determined using isotype-specific goat anti-dog IgG conjugated to horseradish peroxidase (Bethyl Laboratories, Inc., Montgomery, TX). The titer was defined as the highest serum dilutions resulting in an absorbance value 1.0. The error bars stand for standard error. Number of immune serum sample from top to bottom: 8, 10, 16, 5, 5, 5.

Figure 13 depicts representative patterns of immunoblot with naturally exposed dog sera and antisera to experimental vaccines. Naturally exposed dog sera were isolated in New York area. Antisera to experimental vaccine containing 25 μg each FLOspA and FLOspB and 50 μg QS-21 were prepared as described above with respect to Fig. 12. Immunoblot was performed with antigen strips according to the instruction of manufacturer (Cambridge Biotech Corporation, worcester, MA). Serum samples at dilution of 1:50 and goat

anti-dog IgG $F(ab')_2$ conjugated onto horseradish peroxidase (Bethyl Laboratories, Inc., Montgomery, TX) were used to detect the specific antibody. Lane 1, positive serum; lanes 2 to 12, naturally exposed dog sera; lane 13 to 23, antisera; lane 24, negative serum.

Figures 14A and 14B depict comparison of OspA and OspB specific antibody isotype titer of naturally exposed canine sera and antisera to experimental vaccines. Naturally exposed dog sera were described above with respect to Fig. 13. Antisera to experimental vaccine containing 25 μg each of FLOspA and FLOspB and 50 μg of QS-21 were described above with respect to Fig. 12. ELISA was done using plates coated with 0.2 μg OspA and 0.2 μg OspB, respectively. Antibody isotypes were determined using isotype-specific goat anti-dog IgG conjugated to horseradish peroxidase (Bethyl Laboratories, Inc., Montgomery, TX). Titer was defined as described above with respect to Fig. 12.

Figure 15 depicts antiborrelial activity against *B. burgdorferi sensu stricto* strains B31 and CA-2-87 of canine antisera to vaccines formulated with lipidated or nonlipidated OspA and OspB and QS-21. Antisera are described above with respect to Figure 12. Antiborrelial activity of antisera was determined as described in Table 1. Error bars represent the standard deviation of measurements of 8, 10, and 16 serum samples, respectively (from top to bottom).

Figure 16 depicts antiborrelial activity against different geographic Lyme disease spirochetes of *B. burgdorferi sensu stricto* and *B. garinii sp. nov.* of canine antisera to experimental vaccine formulated with 25 μg each of FLOspA and/or FLOspB and with or without QS-21. Antisera preparations are described above with respect to Fig. 12. Antiborrelial activity of antisera was tested as described in Table 1. Error bars represent the standard deviation of measurements of 32, 10, 10, 10, and 3 serum samples, respectively (from top to bottom of legend). High absorbance indicates high antiborrelial activity.

Figure 17 depicts correlation of antiborrelial activity of canine antisera with isotypes IgG1 and IgG2 antibody titer. Antisera to experimental vaccine containing 25 μg each of FLOspA and FLOspB and 50 μg QS-21 are described above with respect to Fig. 12. Antiborrelial activity and IgG antibody titers were determined as described in Table 1 and Fig. 12, respectively. Antiborrelial activity of antisera correlated with IgG2 antibody titer (R=0.61) and not with IgG1 (R=0.13).

Figure 18 depicts expression of recombinant OspA and OspB in *E. coli* strain MZ-1. Bacteria containing recombinant plasmids encoding OspA or OspB were grown to logarithmic phase at 32°C, and then incubated at 42°C for 1 h for induction of recombinant protein synthesis. Whole cell lysates of bacteria before and after induction were analyzed by SDS-PAGE, and visualized by Coomassie blue staining. 1, before induction of MZ-1 harboring pLCBC1 (Beltz, G. A. *et al.*, U.S. Patent No. 4,753,873 (1988)) and *ospA*; 2, after induction; 3, purified OspA; 4, after induction of MZ-1 harboring pLCBC1 and *ospB*; 5, purified OspB. Molecular weight markers (Bio-Rad Laboratories, Melville, New York) in KDa.

Figures 19A, 19B and 19C depict SDS-PAGE and immunoblotting of whole cell lysates of *B. burgdorferi*. Four to 6 μg of lysates of the bacteria were analyzed for protein composition on the 11% SDS-PAGE gel, and visualized by Coomassie blue staining (Fig. 19A). The antigenic properties of OspA and OspB were tested by transferring the separated proteins to the membrane and probing with 1:20 dilution of mouse antisera to OspA (Fig. 19B) or OspB (Fig. 19C) of strain B31. 1, strain B31; 2, strain CA-2-87; 3, strain Fr; 4, strain G25. → indicates an approximately 22 KDa protein band in Fig. 19C. Molecular weight markers are in KDa.

Figure 20 depicts antibody isotype titers of mouse antisera to OspA and OspB formulated with or without either QS-21 or alum. Serially diluted mouse antisera were assayed by ELISA as described (Lindenmayer, J. *et al.*, *J. Clin. Microbiol.* 8:92-96 (1990)). The isotype-specific goat anti-mouse IgG

conjugated to horseradish peroxidase was used to measure antibody isotype. The bars represent group means.

Figure 21 depicts OspA and OspB protein coding regions within *B. burgdorferi*.

Figure 22 depicts the basic features of the expression vector used to express *B. burgdorferi* recombinant OspA and OspB antigens.

Figure 23 depicts the entire DNA sequence of pLCBC1OspA8+6 and the origin of each base. The sequence information was obtained from the following publicly available sources:

1. pBR322 from Genebank Accession # J01749
2. lambda from Genebank Accession # J02459
3. OspA/B from Bergstrom *et al.*, *Mol. Microbiol. 3*:479-486 (1989).

Figure 24 depicts the entire DNA sequence of pLCBC1OspB8+4 and shows the origin of each base. The sequence information was obtained from the following publicly available sources:

1. pBR322 from Genebank Accession # J01749
2. lambda from Genebank Accession # J02459
3. OspA/B from Bergstrom *et al.*, *Mol. Microbiol. 3*:479-486 (1989).

Figure 25 depicts a restriction fragment profile of the OspA clone pLCBC1OspA8+6. From left to right, each lane contains either a control marker or pLCBC1OspA8+6 incubated with the indicated restriction enzyme(s). Lane 1: markers λHindIII + ϕXHaeIII; lane 2: uncut; lane 3: BamHI; lane 4: PvuII; lane 5: EcoRI; lane 6: HindIII; lane 7: NdeI; lane 8: PstI; lane 9: ScaI; lane 10: EcoRI + PvuII; lane 11: EcoRI + HindIII; lane 12: PstI + PvuII; lane 13: λHindIII + ϕXHaeIII markers.

Figure 26 depicts a restriction fragment profile of the OspB clone pLCBC1OspB8+4. From left to right each lane contains either a control marker or pLCBC1OspB8+4 incubated with the indicated restriction enzyme(s). Lane 1: markers λHindIII + ϕXHaeIII; lane 2: uncut; lane 3:

BamHI; lane 4: EcoRI; lane 5: NdeI; lane 6: PstI; lane 7: PvuII; lane 8: BamHI + EcoRI; lane 9: EcoRI + HindIII; lane 10: EcoRI + PstI; lane 11: HindIII + SspI; lane 12: PvuII + SspI; lane 13: markers λHindIII + ϕXHaeIII.

Description of the Preferred Embodiments

The present invention relates to a method for the determination of the bactericidal activity of an antiserum to *B. burgdorferi*, which comprises

(a) contacting said antiserum with a sample containing *B. burgdorferi* and a suitable color pH indicator, such as phenol red, for measurement of bacterial growth; and

(b) measuring the absorbance of the sample;

wherein high absorbance, when compared to a control sample which does not contain said antiserum is an indication of high bactericidal activity.

Color pH indicators are known in the art and may be readily obtained from the various suppliers. For example, various color pH indicators are described in the 1993 catalogue of Sigma Chemical Co., St. Louis, Missouri, at page 1438.

The borreliacidal activity of immune sera to *B. burgdorferi* is an important parameter in the *in vitro* evaluation of protective efficacy of vaccines against Lyme disease (Simon, M.M., *et al.*, *Immunol. Today 12*:11-16 (1991); Schaible, U.E., *et al.*, *Proc. Natl. Acad. Sci. USA 8*:3768-3772 (1990); Kochi and Johnson, *Infect. Immun. 56*:314-321 (1988); Lovrich, S.D., *et al.*, *Infect. Immun. 59*:2522-2528 (1991); Callister, S.M., *et al.*, *J. Clin. Microbiol. 29*:1773-1776 (1991)). Currently used borreliacidal assays examine only a small portion of the sample for viable spirochetes under a dark-field microscope (Kochi and Johnson, *Infect. Immun. 56*:314-321 (1988); Kochi, S.K., *et al.*, *J. Immunol. 146*:3964-3970 (1991); Lovrich, S.D., *et al.*, *Infect. Immun. 59*:2522-2528 (1991); Callister, S.M., *et al.*, *J. Clin. Microbiol. 29*:1773-1776 (1991)). Radioactive incorporation assays and

spirochete culture are used to verify the results (Kochi and Johnson, *Infect. Immun. 56*:314-321 (1988); Pavia, C.S., *et al.*, *J. Infect. Dis. 163*:656-659 (1991); Lovrich, S.D., *et al.*, *Infect. Immun. 59*:2522-2528 (1991); Callister, S.M., *et al.*, *J. Clin. Microbiol. 29*:1773-1776 (1991)). It is apparent that the existing bactericidal assays lack simplicity, reliability, and cannot handle a large number of serum samples.

The present microtiter CBA uses phenol red as an indicator of the accumulation of nonvolatile acid generated by spirochete metabolism. The CBA detects borreliacidal activity of immune sera by measuring the absorbance at 562/630 nm. Significant decrease in absorbance represents the bacterial survival and growth; a small decrease in absorbance indicates borrelial killing. To increase the sensitivity of the colorimetric assay, a relatively high concentration of phenol red (final concentration 60 μg/ml) was required. The dual wavelengths of 562/630 nm most sensitively reflected the absorbance changes in the presence of acid. To evaluate the validity of the colorimetric assay, both the TIA and colorimetric assay using serially diluted spirochetes or using a concentration of 4 x 10^6 spirochetes and serially diluted antisera to lysates of strain B31 were performed. Correlation coefficients of these two assays were 0.977 and 0.935, respectively, and were linearly related over the useful range of most microplate readers.

An increase in the absorbance of the first several serum dilutions after 24 h incubation, compared with the absorbance before incubation, was consistently seen (Fig. 3). This change could have been caused by the dissociation of CO_2 from the plate during the incubation. The slow growth of the spirochete and borreliacidal activity of the antisera might have prevented the production of the enough acid products to reduce pH.

The absorbance of the borreliacidal mixture with each serum dilution decreased at a constant rate during 48 h to 96 h incubation (Fig. 3). After 48 h incubation, the inhibition of borrelial growth by the antisera was clearly detectable, as shown by the absorbance. The inhibition of the borrelial growth

(significantly less absorbance change) was clearly dependent on the concentration of antisera (Figs. 3 and 4).

The mouse antisera to lysates of strain B31 had a high borreliacidal activity against the homologous strain B31 (Fig. 4), strains Cr (a Wisconsin tick isolate), and Fr (a German tick isolate), but had little borreliacidal activity against the Swedish strain G25, a different genomic species in terms of rRNA gene restriction patterns (Postic, D. *et al.*, *Res. Microbiol. 141*:465-475 (1990)). Similarly, the antisera to strain G25 possessed a high borreliacidal activity against the homologous strain G25, but did not against other strains tested. Normal mouse sera, compared with mBSK control, were not borreliacidal (Fig. 4). Dark-field microscopy showed that the spirochetes were killed or lysed by the antisera, but not by the normal mouse sera. Thus, the inhibition of absorbance changes in the CBA was because of the borreliacidal activity of the specific antibody. The turbidity increase due to the borrelial growth was negligible and did not significantly influence the absorbance changes.

In the presence of a high concentration of the antisera, approximately 10-20% of the spirochetes still survived. This observation was consistent with previous reports that human convalescent sera or rat immune sera fail to kill all spirochetes *in vitro* (Pavia, C.S., *et al.*, *J. Infect. Dis. 163*:656-659 (1991); Lovrich, S.D., *et al.*, *Infect. Immun. 59*:2522-2528 (1991); Callister, S.M., *et al.*, *J. Clin. Microbiol. 29*:1773-1776 (1991)).

Both the CBA and TIA showed the antisera to strain B31 without complement killed the spirochete as efficiently as those containing guinea pig complement. Human or hamster immune sera, however, do require complement for killing *B. burgdorferi* (Kochi and Johnson, *Infect. Immun. 56*:314-321 (1988); Kochi, S.K., *et al.*, *J. Immunol. 146*:3964-3970 (1991); Lovrich, S.D., *et al.*, *Infect. Immun. 59*:2522-2528 (1991)), while heat-inactivated rat antisera to *B. burgdorferi* or monoclonal antibody to a surface epitope of the spirochete in the absence of complement source are also borreliacidal (Pavia, C.S., *et al.*, *J. Infect. Dis. 163*:656-659 (1991); Coleman

and Benach, "Characterization of antigenic determinants of *Borrelia burgdorferi* shared by other bacteria," *J. Infect. Dis. 165*:658-666 (1992)). Classically, complement components are required for the destruction of the Gram-negative bacteria, including *B. burgdorferi*, by bactericidal antibody.

To quantitate the borreliacidal activity of immune sera, the borreliacidal titer was defined as the highest dilution of sera which inhibits absorbance change caused by 50% (2 x 10^6 bacteria) of spirochetes in the CBA.

In summary, the microtiter CBA for determination of borreliacidal activity of immune serum has been developed with the advantages of simplicity, reliability, and safety. Furthermore, the CBA can handle a large number of serum samples and is valuable in Lyme vaccine development and as a diagnostic method for Lyme borreliosis.

Thus, the invention also relates to a method for the detection of Lyme borreliosis, comprising

(a) contacting an antiserum against *B. burgdorferi* with a suitable color pH indicator, such as phenol red, for measurment of bacterial growth, and a sample suspected of containing *B. burgdorferi*; and

(b) measuring the absorbance of the sample;

wherein high absorbance, when compared to a control sample which does not contain *B. burgdorferi*, is an indication that *B. burgdorferi* is present in said suspected sample.

Color pH indicators are known in the art and may be readily obtained from the various suppliers. For example, various color pH indicators are described in the 1993 catalogue of Sigma Chemical Co., St. Louis, Missouri, at page 1438.

The invention also relates to a vaccine, comprising OspA, OspB or fragments thereof; and a saponin adjuvant.

Investigation of immunoprotective antigens of Lyme disease spirochetes and development of Lyme disease vaccine have been the focus of Lyme disease research (Fikrig *et al., Science 250*:553-6 (1990); Fikrig *et al., Infect.*

Immun. 60:657-661 (1992); Edelman, R., *Vaccine 9*:531-2 (1991); Fikrig *et al., Proc. Natl. Acad. Sci. USA 89*:5418-21 (1992)). OspA or OspB based experimental vaccines formulated with Freund's adjuvants have been shown to confer protection against challenge by either syringe injection or spirochete infected tick feeding in mouse model (Fikrig *et al., Infect. Immun. 60*:657-661 (1992); Fikrig *et al., Proc. Natl. Acad. Sci. USA 89*:5418-21 (1992)). Although these results are encouraging, it is important to determine the influence of clinically acceptable adjuvants on the immunogenicity of the recombinant proteins. Hence, the inventors have identified and purified a potent saponin adjuvant, QS-21, from *Quillaja saponaria* (Kensil, C. R. *et al., J. Immunol. 146*:431-437 (1990)). This adjuvant significantly enhances both humoral and cellular immune responses to a variety of antigens and has been used in recombinant subunit vaccines including the experimental HIV-1 gp160 protein vaccine and commercially available feline leukemia virus vaccine (Marciani D. J. *et al., Vaccine 9*:89-96 (1991); Newman, M. J. *et al., J. Immunol. 148*:2357-2362 (1992); Wu, J.-Y. *et al., J. Immunol. 148*:1519-1525 (1992)). The impact of QS-21 and aluminum hydroxide (alum) on the functional antibody responses to the recombinant OspA and OspB derived from *B. burgdorferi* strain B31 was determined in mice. Only QS-21 induced high titers of IgG2a and IgG2b antibodies, the complement fixing isotypes (Kochi, S. K. *et al., J. Immunol. 146*:3964-3970 (1991); Schmitz, J. L. *et al., Infect. Immun. 60*:2677-2682 (1992); Spiegelberg, H. L., *Adv. Immunol. 19*:259-294 (1974)). QS-21 was shown to be superior to alum in enhancing functional antibody response to OspA and OspB. The experimental vaccine containing OspA and OspB formulated with QS-21 conferred complete protection against infection with either the homologous or the heterologous strains of *B. burgdorferi* in mice.

Hence, according to the obtained results a saponin adjuvant, QS-21, significantly enhances immunogenicity of OspA and OspB. In contrast, aluminum hydroxide, a widely used adjuvant for human vaccines, does not significantly influence OspA or OspB based experimental Lyme vaccines. As

mentioned above, the impact of the adjuvants QS-21 and aluminum hydroxide (alum) on the immunogenicity of recombinant outer surface protein A (OspA) and B (OspB) of *Borrelia burgdorferi* was investigated. Both nonacylated OspA and OspB derived from strain B31 were expressed in *Escherichia coli* and purified by reversible citraconylation and anion exchange chromatography. Antisera to OspA or OspB were prepared in mice with antigens formulated with QS-21 or alum, and evaluated for specific immunoglobulin G isotypes, agglutination, and borreliacidal activity. QS-21 significantly enhanced IgG2a and IgG2b antibody responses to OspA and OspB, and IgG1 response to OspA when compared with the formulation containing antigen alone. In contrast, alum significantly inhibited the induction of IgG2a and IgG2b responses to OspA. Alum had no significant effect on IgG1 response to OspA, or IgG2a and IgG2b responses to OspB, but significantly enhanced IgG1 antibody response to OspB. Antisera to OspA or OspB formulated with QS-21 possessed higher titers of agglutinating antibody than antisera to OspA or OspB alone, respectively. Borreliacidal activity was 8- to 64-fold higher in antisera to OspA formulated with QS-21 than in antisera to OspA formulated with or without alum. These antisera were highly borreliacidal to *B. burgdorferi*, New York strain B31, a California isolate CA-2-87, German isolate Fr, and Swedish *B. garinii* species, G25. Antisera to OspB formulated with QS-21 were highly borreliacidal to strains B31 and Fr, but not to CA-2-87 and G25. Antisera to OspB formulated with alum were borreliacidal only to B31. Thus, OspA was superior to OspB and QS-21 superior to alum at eliciting functional antibody responses. The vaccine containing OspA and OspB formulated with QS-21 was protective in mice against infection with 10^5 infectious spirochetes of strains B31 or CA-2-87.

As mentioned above, experimental vaccines containing OspA and Freund's adjuvant can also eliminate *B. burgdorferi* from challenge ticks feeding on the immunized mice (Fikrig, E. *et al.*, *Proc. Natl. Acad. Sci. USA* *89*:5418-5421 (1992)). It has been shown that *in vivo* immunoprotection is correlated with *in vitro* borreliacidal activity of immune serum to *B.*

burgdorferi (Lovrich, S. D. *et al.*, *Infect. Immun.* 59:2522-2528 (1991)). As part of the present invention, we studied and, hence, determined the role of the adjuvants QS-21 and alum on enhancement of functional antibody responses to recombinant OspA and OspB of *B. burgdorferi*.

Both recombinant OspA and OspB were not acylated and were freely soluble proteins. They were monomeric at the concentrations used. The dose of QS-21 used in the vaccine formulation was below its critical micellar concentration (Kensil, C. R. *et al.*, *Vaccine Res.* 2:273-281 (1993). The possibility exists that these antigens and QS-21 formed soluble lipid-protein complexes. This is fundamentally different from the antigens adsorbed onto an insoluble matrix such as alum.

Mice immunized with Osp vaccine formulated without adjuvant raised significantly higher titers of antibody to OspA than to OspB, indicating OspA was more immunogenic than OspB. In general, mice immunized with OspA or OspB formulated with QS-21 raised significantly higher antibody responses than those immunized with the antigens alone or antigens formulated with alum. These antisera also possessed higher agglutination antibody titers and borreliacidal activity. It appeared that the borreliacidal activity of the antisera was correlated with the agglutination antibody titer and ELISA antibody titer with the exception of the low agglutination titer with strain G25.

Antisera to OspA formulated with QS-21 had a higher titer of agglutinating antibody than antisera to OspB formulated with QS-21. Those antisera reacted with not only strain B31, but also the heterologous California isolate CA-2-87, German isolate Fr, and Swedish isolate G25. Similarly, antisera to OspA formulated with QS-21 had significantly higher borreliacidal activity against the 4 strains than antisera to OspB formulated with QS-21. These results showed that OspA induced higher functional antibody responses than OspB. In addition, antisera to OspB formulated with QS-21 also recognized an approximately 22 KDa protein band of strains B31, CA-2-87, and Fr, but not strain G25. This OspB related lower molecular mass protein may be similar to a 21 KDa protein that Bundoc and Barbour have previously

observed (Bundoc & Barbour, *Infect. Immun 57*:2733-2741 (1989)). Recently, a premature stop codon in the *ospB* gene that terminates OspB 58 amino acids short of the full length protein has been observed by Rosa *et al.* (Rosa, P. A. *et al.*, *Mol. Microbiol 6*:3031-3040 (1992)). They indicate that this lower molecular mass protein may be a smaller OspB fragment.

SDS-PAGE and immunoblotting analyses showed that OspA and OspB of strain G25 and OspB of the California isolate CA-2-87 were different from those of strain B31 and the German isolate Fr based on their migration rate. However, antisera to B31 OspA and OspB reacted with OspA and OspB of strains G25, CA-2-87, and Fr. This observation was similar to a previous report that antiserum to recombinant OspA of strain B31 reacts with OspA proteins of other strains including some European strains (Milch & Barbour, *J. Infect. Dis. 160*:351-353 (1989)). Recently, the strains B31 and G25 were designated as OspA serotypes 1 and 6, respectively, based on an OspA serotyping system (Wilske, B. *et al.*, *J. Clin. Microbiol 31*:340-350 (1993)). Three genospecies of borrelial isolates have been proposed; strains B31 and G25 are included in *B. burgdorferi sensu stricto* and *B. garinii sp. nov.*, respectively (Milch, L. *et al.*, *J. Infect. Dis. 160*:351-353 (1989)). QS-21 significantly increased the agglutination antibody and borreliacidal activity against borrelial isolates with different OspA serotypes or genospecies. This functional increase in antisera may have resulted from a broadening of antibody response to outer surface exposed epitopes on OspA and OspB. This should be kept in mind when developing an effective Lyme disease vaccine because at least seven different OspA serotypes of *B. burgdorferi* have recognized thus far (Wilske, B. *et al.*, *J. Clin. Microbiol 31*:340-350 (1993)).

The major antibody isotypes enhanced by QS-21 in mice were IgG2a and IgG2b. Alum increased only the IgG1 antibody response, consistent with a previous report (Byars, N. E. *et al.*, *Vaccine 9*:309-318 (1991)). This characteristic of QS-21 in enhancing IgG isotypes is important because the isotypes of IgG antibody differ in immunoprotective efficacy for many infectious diseases (Spiegelberg, H. L., *Adv. Immunol. 19*:259-294 (1974);

Briles, D. E. *et al.*, *J. Mol. Cell. Immunol. 1*:305-309 (1984); Coutelier, J. P. *et al.*, *J. Exp. Med. 168*:2373-2378 (1988)). Complement fixation (Kochi, S. K. *et al.*, *J. Immunol. 146*:3964-3970 (1991); Schmitz, J. L. *et al.*, *Infect. Immun. 60*:2677-2682 (1992); Spiegelberg, H. L., *Adv. Immunol. 19*:259-294 (1974)); and efficient opsonization by IgG2a and IgG2b (Benach, J. L. *et al.*, *J. Infect. Dis. 150*:497-507 (1984); Unkeless & Eisen, *J. Exp. Med. 142*:1520-1533 (1975)) may enhance protective mechanisms against borrelial infection. Human or hamster immune sera require complement for killing *B. burgdorferi* (Kochi, S. K. *et al.*, *J. Immunol. 146*:3964-3970 (1991); Schmitz, J. L. *et al.*, *Infect. Immun. 60*:2677-2682 (1992)), while mouse OspB-specific monoclonal antibody IgG1 can also kill spirochetes in the absence of complement (Coleman, J. L. *et al.*, *Infect. Immun. 60*:3098-3104 (1992)). Thus, *B. burgdorferi* may be killed in both complement-dependent and complement-independent fashions. It has been reported, however, that OspA-specific monoclonal antibody IgG2b confers better passive protection against experimental diseases than OspA-specific monoclonal antibody IgG1 (Schaible, U. E. *et al.*, *Proc. Natl. Acad. Sci. USA 8*:3768-3772 (1990)). Hamster IgG2 from immune sera conferred complete passive protection to irradiated hamsters from experimental borrelial infection in a complement-dependent fashion (Schmitz, J. L. *et al.*, *Infect. Immun. 60*:2677-2682 (1992)). Thus, complement-mediated killing may be a significant and efficient protection mechanism *in vivo*. QS-21 also significantly induced OspA and OspB specific antibody IgG1 and IgG2 responses in dogs and the borreliacidal activity appeared to be associated with IgG2. Thus, QS-21 serves as an important component in a vaccine against Lyme disease.

OspA or OspB formulated with Freund's adjuvant (Fikrig, E. *et al.*, *Science 250*:553-556 (1990); Fikrig, E. *et al.*, *Infect. Immun. 60*:657-661 (1992); Fikrig, E. *et al.*, *Proc. Natl. Acad. Sci. USA 89*:5418-5421 (1992)) or OspA formulated with alum (Erdile, L. F. *et al.*, *Infect. Immun. 61*:81-90 (1993)) have been shown to confer protection against experimental challenge in mice. The present study indicated that QS-21 was more potent than alum

in enhancing the immunogenicity of OspA and OspB. Antibody to OspA formulated with QS-21 was borreliacidal to all the strains tested, and antibody to OspB formulated with QS-21 borreliacidal to strains B31 and Fr. Some borrelial isolates do not produce OspA or OspB. Thus, the vaccine containing both OspA and OspB may provide better and broader protection. Our *in vivo* study showed that the vaccine containing OspA, OspB, and QS-21 was capable of conferring protection against the infection of the high doses of the homologous and heterologous spirochetes.

QS-21 has been used in a commercially available feline leukemia virus vaccine (Marciani, D. J. *et al.*, *Vaccine* 9:89-96 (1991)) and in an experimental HIV-1 vaccine in *rhesus macaques* (Livingston, P. O., *Annals New York Acad. Sci. 690*:204-213 (1993)). This adjuvant has been shown to significantly enhance both antibody and cell-mediated immune responses and has little or no toxicity in laboratory animals (Kensil, C. R. *et al.*, *J. Immunol. 146*:431-437 (1990); Marciani D. J. *et al.*, *Vaccine* 9:89-96 (1991); Newman, M. J. *et al.*, *J. Immunol. 148*:2357-2362 (1992); Livingston, P. O., *Annals New York Acad. Sci. 690*:204-213 (1993)). A human phase I melanoma ganglioside vaccine trial has shown that a QS-21 formulated GM2-KLH vaccine induces significantly higher antibody responses than other adjuvant formulated vaccines without associated toxicity. Therefore, QS-21 is a useful adjuvant for both veterinary and human Lyme vaccine.

In summary, OspA and OspB of *B. burgdorferi* possessed borreliacidal epitopes, and the adjuvant QS-21 was more efficient than alum in inducing antibody responses to OspA and OspB. QS-21 significantly enhanced IgG2a, IgG2b, and functional antibody responses, and as shown below, it is an important component of a Lyme disease vaccine. The experimental vaccine containing OspA and OspB formulated with QS-21 was shown to be highly protective in mice and as shown below, was found to be highly effective in the prevention of Lyme disease in dogs.

TABLE 1. **Agglutination with divergent borrelial strains of mouse antisera to OspA and OspB formulated with or without QS-21**

Immunogens	Agglutination titer[1]			
	Strain B31	CA-2-87	G25	Fr
OspA	1,600	800	≤ 50	400
OspA + alum	800	800	100	800
OspA + QS-21	6,400	1,600	400	1,600
OspB	200	100	≤ 50	100
OspB + alum	400	400	≤ 50	400
OspB + QS-21	400	200	≤ 50	400
Normal mouse serum	≤ 50	≤ 50	≤ 50	≤ 50

[1] Microagglutination of mouse antisera to OspA or OspB with different geographic isolates of *B. burgdorferi* was performed by incubating 100 μl of *B. burgdorferi* (approximately 1 x 10^6 spirochetes) with an equal volume of heat-inactivated, serially diluted antisera in mBSK medium at 32°C for 2 hours. Agglutination was determined by dark-field microscopy, and its titer defined as the highest dilution of the sera that caused ≥ 50% of the spirochetes to agglutinate.

TABLE 2. **Borreliacidal Activity against divergent borrelial strains of mouse antisera to OspA and OspB formulated with or without adjuvants**

Immunogens	Borreliacidal titer[1]			
	Strain B31	CA-2-87	G25	Fr
OspA	80	≤ 10	40	40
OspA + alum	80	≤ 10	80	80
OspA + QS-21	1,280	640	640	320
OspB	20	≤ 10	≤ 10	≤ 10
OspB + alum	320	≤ 10	≤ 10	≤ 10
OspB + QS-21	320	≤ 10	≤ 10	160
Normal mouse serum	≤ 10	≤ 10	≤ 10	≤ 10

[1] Borreliacidal activity of mouse antisera to OspA and OspB against different geographic isolates of *B. burgdorferi* was determined by the colorimetric assay of the present invention as described below. Serially diluted, heat-inactivated sera in mBSK containing 120 μg of phenol red per ml were incubated with spirochetes (approximately 4 x 10^6 of spirochetes) and complement source in 96-well microliter plates. After 48 to 96 h incubation at 32°C, the absorbance at 562/630 nm was measured using a microplate reader. The titer of borreliacidal activity was defined as the highest dilution of the antisera which inhibited absorbance change caused by 50% (2 x 10^6 bacteria) of spirochetes used in this assay.

Further, humoral immune responses to experimental Lyme disease Osp subunit vaccines and to natural borrelial infection in dogs were investigated by characterization of immune sera and naturally exposed dog sera isolated in the New York area. The experimental subunit vaccines were formulated with adjuvant QS-21, OspA and/or OspB. Beagles were subcutaneously vaccinated twice at 12 and 16 weeks of age, and two weeks after the second vaccination immune sera was isolated for analysis of immunoglobulin (Ig) G isotype antibody responses and antiborrelial activity. QS-21 formulated vaccine containing liproproteins OspA and OspB elicited 4-fold higher IgG1 ($p<0.1$) and 8-fold higher IgG2 ($p<0.05$) antibody responses than non-QS-21 formulated vaccines. Nonlipidated OspA and OspB based vaccine formulated

with QS-21 elicited significantly ($p < 0.005$) lower IgG1 and IgG2 antibody titer than lipoproteins OspA and OspB based vaccine. Antisera induced by the vaccine containing QS-21, OspA and OspB possessed high titer of antibodies to both proteins. Antisera induced by either OspA or OspB based vaccine had high titers of antibody and antiborrelial activity against *B. burgdorferi sensu stricto* strains B31 and CA-2-87 as those elicited by OspA and OspB based vaccine. However, only the latter were also antiborrelial to heterologous borrelial strain 24008 Fr and *B. garinii sp. nov.* strain G25, both European isolates. Antisera to vaccine formulated with lipoproteins OspA and OspB possessed significantly ($p < 0.005$) higher antiborrelial activity than those to vaccine formulated with nonlipidated OspA and OspB. Among 63 naturally exposed *B. burgdorferi* specific antibody positive dog sera, 24 (38%) sera had antibodies to both OspA and OspB; 3 (5%) and 4 (6%) sera had antibody to only OspA and OspB, respectively. Titers of OspA and OspB specific antibodies were low. Thirteen of 63 (21%) sera had low titer of antiborrelial activity; 7 of these 13 sera had antibodies neither to OspA nor to OspB. Thus, (1) immunogenicity of OspA and OspB based subunit vaccine can be greatly enhanced using lipoproteins OspA and OspB; (2) immunogenicity of Osp subunit vaccine can be further enhanced by the adjuvant QS-21; (3) OspA and OspB based vaccine was more potent than a single protein based vaccine in eliciting functional humoral immune response in dogs.

This study demonstrated that a QS-21 formulated, FLOspA and FLOspB based vaccine was able to elicit maximal humoral immune response in dogs. The enhanced immune response may have resulted from the double adjuvant activity of the lipid component of the full length outer surface proteins and QS-21. The lipid structure of OspA has been suggested to possess adjuvant activity and is required for enhanced immunogenicity of OspA in mice (Erdile *et al.*, *Infect. Immun.* *61*:81-90 (1993)). This is consistent with the present observation that in the formulation with QS-21, lipidated OspA and OspB were significantly ($p < 0.005$) more immunogenic than nonlipidated OspA and OspB, and induced significantly ($p < 0.005$) higher

functional antibody response in dogs. As described above, QS-21 is superior to aluminum hydroxide in enhancing humoral immune response to truncated nonlipidated OspA and OspB in mice. Aluminum hydroxide is incapable of enhancing antibody response to FLOspA (Erdile *et al., Infect. Immun. 61*:81-90 (1993)). However, the present data clearly demonstrated that QS-21 was also capable of significantly enhancing humoral immune response to FLOspA and FLOspB in dogs. Moreover, QS-21 induced broadening of isotype antibody responses in dogs as in mice, which will enhance host immunity by enhancing bactericidal and opsonophagocytic activity against spirochetal infection (Benach *et al., J. Infect. Dis. 150*:497-507 (1984); Kochi *et al., Infect. Immun. 56*:314-21 (1988), Peterson *et al., Infect. Immun. 46*:608-11 (1984).

The present data showed that FLOspA and FLOspB based vaccine formulated with QS-21 was much more potent than either FLOspA or FLOspB based vaccine in inducing functional immune response. OspA or OspB based, QS-21 formulated vaccine was capable of inducing an antibody response which was antiborrelial against an homologous *B. burgdorferi sensu stricto* strain B31 and a heterologous strain CA-2-87 (a California isolate), but not against two European isolates strain 24008 Fr (a French isolate) and *B. garinii sp. nov.* strain G25 (a Swedish isolate). However, OspA and OspB based, QS-21 formulated vaccine elicited antibodies which were also antiborrelial to these two European isolates. This indicated that weakly immunogenic, antiborrelial epitope(s) may be conserved on OspA and OspB among these strains, and that antibodies to these epitopes may have had synergistic antiborrelial action on spirochetes. At least three genospecies and six different OspA serogroups of Lyme disease spirochetes have been recognized (Baranton *et al., Int. J. Sys. Bacteriol. 42*:378-83 (1992); Wilske *et al., J. Clin. Microbiol. 31*:340-50 (1993)). Strains B31 and G25 have been designated as OspA serogroups 1 and 6, respectively (Wilske *et al., J. Clin. Microbiol. 31*:340-50 (1993)). OspA and OspB of strains B31, CA-2-87, G25 have different electrophoresis profile (see Figures 19A-19C). Cross antiborrelial activity against different

genospecies and OspA serogroups of spirochetes of the antisera elicited by the experimental vaccine showed promise for developing an effective vaccine against Lyme disease for different geographic regions. The high functional activity of antisera elicited by QS-21 formulated vaccine may have resulted from enhanced antibody titer and broadening antibody responses to functional epitopes on OspA and OspB of the spirochete. Antiborrelial activity of canine antisera appeared to be associated with isotype IgG2 antibody (R=0.61), not with IgG1 antibody (R=0.13). This may be due to higher titer of IgG2 antibody responses elicited by the vaccine.

It has been reported that dogs naturally infected by Lyme disease spirochete did not have detectable antibodies to OspA (Greene *et al., J. Clin. Microbiol. 26*:648-53 (1988)). In the present study, 63 naturally exposed canine sera have been identified which have specific antibodies to Lyme disease spirochete. Forty-nine percent of these sera had antibodies to OspA and/or OspB, which is inconsistent with the previous report (Greene *et al., J. Clin. Microbiol. 26*:648-53 (1988)). The serum samples of the present invention were isolated from Westchester and Long Island, New York, a highly endemic region for Lyme disease (Alpert *et al., NY State J. Med. 92*:5-8 (1992)). Repeat infection of dogs by Lyme disease spirochetes in this area may explain the different observations. Although relatively large number of sera samples contained OspA and OspB specific antibodies, their titers were low. It has been suggested that the quality of immune response to OspA and OspB is dependent on the antigen load (Schaible *et al., Immunol. Let. 36*:219-26 (1993)). This poor antibody response to natural borrelial infection may be related to the quantity of spirochetes delivered by tick bite. In addition, only 13/63 (21%) naturally exposed sera had detectable antiborrelial activity, and 7 of them did not have antibodies to OspA and OspB, indicating antibodies to other than OspA and OspB are also antiborrelial. These protective antigens may be candidates for vaccines.

In summary, QS-21 formulated, lipoproteins OspA and OspB based experimental vaccines were able to elicit high humoral immune response in

dogs regardless of the fact that the natural borrelial infection stimulates poor antibody responses to the protective immunogens. The immune sera were highly antiborrelial to both homologous and heterologous Lyme disease spirochetes. The present experimental vaccines have also conferred protection from spirochete infected tick challenge of dogs, thus showing potential for the development of subunit vaccines for dogs and humans against Lyme disease.

Table 3. OspA and OspB specific antibody and antiborrelial activity of naturally exposed, seropositive dog sera.

Immunoblot band	Reaction	Serum no.	Antiborrelial titer
OspA and OspB	+[a]	6	10 to 160
OspA and OspB	+	18	-[b]
OspA and OspB	-	7	10 to 80
OspA and OspB	-	25	-
OspA only	+	3	-
OspB only	+	4	-

+[a]: Positive; -[b]: Negative.

Immunoblotting was performed as described in Fig. 13. Antiborrelial activity of sera was determined according to the method of the present invention. Heat-inactivated, filter-sterilized sera were serially diluted in 100 μl of mBSK containing 120 μg of phenol red per ml in duplicate in 96-well plates. Five μl of sterile guinea pig complement and 95 μl of spirochete (containing approximately 4 x 10^6 of spirochetes) of strain B31 in fresh mBSK were added to each well. After 48 h incubation at 32°C, the absorbance at 562/630 nm was measured using a microplate reader. The titer of borreliacidal activity was defined as the highest dilution of the antisera which inhibits absorbance change caused by 50% (2 x 10^6 bacteria) of spirochetes used in the assay.

The proteins OspA and OspB and fragments thereof may be obtained as described in the present specification or according to any other methods known in the literature. *See*, for example, Erdile, L. F. *et al.*, *Infect. Immun.* *61*:81-90 (1993); Fikrig, E. *et al.*, *Science 250*:553-556 (1990); Bergstrom, S. *et al.*, *Mol. Microbiol. 3*:479-86 (1989); Howe, T.R. *et al.*, *Science 227*:645-6 (1985); Rosa, P.A. *et al.*, *Mol. Microbiol. 6*:3031-40 (1992); Simon, M.M *et al.*, *J. Infect. Dis. 164*:123-32 (1991), the contents of which are fully incorporated by reference herein.

The terms "OspA" or "OspB" as used herein include lipidated and non-lipidated as well as acylated and non-acylated forms of the outer surface proteins A and B, unless indicated otherwise.

The term "saponin" as used herein includes glycosidic triterpenoid compounds which produce foam in aqueous solution, have hemolytic activity in most cases, and possess immune adjuvant activity. The invention encompasses the saponin *per se*, as well as natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives. The term "saponin" also encompasses biologically active fragments thereof.

Adjuvant saponins have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria Molina*. *See*, U.S. Patent No. 5,057,540, the contents of which are fully incorporated by reference herein. At least 22 peaks with saponin activity were separable. The predominant purified *Quillaja* saponins have been identified as QS-7, QS-17, QS-18, and QS-21. These saponins have been purified by high pressure liquid chromatography (HPLC) and low pressure silica chromatography. These four saponins have adjuvant effect in mice. QS-21 was further purified using hydrophilic interaction chromatography (HILIC) and resolved into two peaks, QS-21-V1 and QS-21-V2, which have been shown to be different compounds. These saponins are useful as immune adjuvants and enhance immune responses in individuals at a much lower concentration than the previously available heterogeneous saponin preparations without the toxic effects associated with crude saponin preparations.

"QS-21" designates the mixture of components QS-21-V1 and QS-21-V2 which appear as a single peak on reverse phase HPLC on Vydac C4 (5 μm particle size, 330 Å pore, 4.6 mm ID x 25 cml) in 40 mM acetic acid in methanol/water (58/42, v/v). The component fractions are referred to specifically as QS-21-V1 and QS-21-V2 when describing experiments or results performed on the further purified components.

In the preferred embodiment, the saponins of the present invention are purified from *Quillaja saponaria Molina* bark. Aqueous extracts of the *Quillaja saponaria Molina* bark were dialyzed against water. The dialyzed extract was lyophilized to dryness, extracted with methanol and the methanol-soluble extract was further fractionated on silica gel chromatography and by reverse phase high pressure liquid chromatography (RP-HPLC). The individual saponins were separated by reverse phase HPLC as described in Example 1. At least 22 peaks (denominated QS-1 to QS-22) were separable. Each peak corresponded to a carbohydrate peak and exhibited only a single band on reverse phase thin layer chromatography. The individual components were identified by retention time on a Vydac C_4 HPLC column as follows:

Peak	Retention Time (minutes)
QS-1	solvent front
QS-2	4.6
QS-3	5.6
QS-4	6.4
QS-5	7.2
QS-6	9.2
QS-7	9.6
QS-8	10.6
QS-9	13.0
QS-10	17.2
QS-11	19.0
QS-12	21.2
QS-13	22.6
QS-14	24.0
QS-15	25.6
QS-16	28.6
QS-17	35.2
QS-18	38.2

QS-19	43.6
QS-20	47.6
QS-21	51.6
QS-22	61.0

The purified saponins are characterized by carbohydrate content, reverse phase and normal phase TLC, UV, infra red, NMR spectra, and fast atom bombardment - mass spectroscopy.

The approximate extinction coefficient determined for 1% (w/v) solutions in methanol at 205 nm of several of the more preferred purified saponins are as follows:

	$\underline{1\%\ E_{205\ nm}}$
QS-7	34
QS-17	27
QS-18	27
QS-21	28

Carbohydrate content was used to quantitate the saponins in some instances. The carbohydrate assay was the anthrone method of Scott and Melvin (*Anal. Chem. 25*:1656 (1953)) using glucose as a standard. This assay was used to determine a ratio of extent of anthrone reaction (expressed in glucose equivalents) per mg of purified saponin (dry weight) so that dry weight of a particular preparation could be estimated by use of anthrone assay. It must be noted that differences in reactivity with anthrone for different saponins may be due to carbohydrate composition rather than quantity as different monosaccharides react variably in this assay.

The substantially pure QS-7 saponin is characterized as having immune adjuvant activity, containing about 35% carbohydrate (as assayed by anthrone) per dry weight, having a uv absorption maxima of 205-210 nm, a retention time of approximately 9 - 10 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 52-53% methanol from a Vydac C_4 column having 5 μm particle

size, 330 Å pore, 10 mM ID X 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately .06% in water and .07% in phosphate buffered saline, causing no detectable hemolysis of sheep red blood cells at concentrations of 200 μg/ml or less, and containing the monosaccharide residues terminal rhamnose, terminal xylose, terminal glucose, terminal galactose, 3-xylose, 3,4-rhamnose, 2,3-fucose, and 2,3-glucuronic acid, and appose (linkage not determined).

The substantially pure QS-17 saponin is characterized as having adjuvant activity, containing about 29% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 35 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol-water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 63-64% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm ID x 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of .06% (w/v) in water and .03% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at 25 μg/ml or greater, and containing the monosaccharide residues terminal rhamnose, terminal xylose, 2-fucose, 3-xylose, 3,4-rhamnose, 2,3-glucuronic acid, terminal glucose, 2-arabinose, terminal galactose and appose (linkage not determined).

The substantially pure QS-18 saponin is characterized as having immune adjuvant activity, containing about 25-26% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 38 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 64-65% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm ID x 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical

micellar concentration of .04% (w/v) in water and .02% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at concentrations of 25 μg/ml or greater, and containing the monosaccharides terminal arabinose, terminal appose, terminal xylose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose, and 2,3-glucuronic acid.

The substantially pure QS-21 saponin is characterized as having immune adjuvant activity, containing about 22% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 51 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 69 to 70% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm x ID 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, with a critical micellar concentration of about .03% (w/v) in water and .02% (w/v) in phosphate buffered saline, and causing hemolysis of sheep red blood cells at concentrations of 25 μg/ml or greater. The component fractions, substantially pure QS-21-V1 and QS-21-V2 saponins, have the same molecular weight and identical spectrums by FAB-MS. They differ only in that QS-21-V1 has a terminal appose which is xylose in QS-21-V2 (which therefore has two terminal xyloses and no appose). The two components additionally contain the monosaccharides terminal arabinose, terminal appose, terminal xylose, 4-rhamnose, terminal galactose, 2-fucose, 3-xylose, and 2,3-glucuronic acid.

As described in the present specification, QS-21 significantly enhances the immunogenicity of truncated OspA (TOspA) and OspB (TOspB) of *B. burgdorferi sensu stricto* strain B31 and broadens immunoglobulin (Ig) G antibody responses in mice. Further, the immunological properties of QS-21 formulated, OspA and/or OspB based experimental Lyme disease vaccines were characterized in dogs. The data showed that QS-21 significantly enhanced antibody response to the experimental vaccines. Vaccine elicited

antisera had high titer of antiborrelial activity. Only the experimental vaccine containing both full length OspA (FLOspA) and OspB (FLOspB) induced antibodies which were antiborrelial not only to the homologous strain, but also to the heterologous and different genospecies of Lyme disease spirochetes.

The vaccines of the invention are useful as vaccines which induce active immunity toward antigens in individuals. Preferably, such individuals are humans, however the invention is not intended to be so limiting. Any animal which may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed invention.

The vaccines of the present invention induce active immunity when administered over a wide range of dosages and a wide range of ratios to the antigen being administered. In one embodiment, the saponin is administered in a ratio of adjuvant to OspA/OspB (w/w) of 3.0 or less, preferably 1.0 or less.

The OspA, OspB and saponin may be administered either individually or admixed with other substantially pure adjuvants to achieve the enhancement of the immune response. Moreover, the vaccines of the present invention may comprise a single saponin or mixtures of saponins. The mixtures of the saponins may be purified saponins or crude mixtures of saponins.

Among the saponin mixtures effective in the present invention are fractions QS-7 and QS-17, QS-7 and QS-18, QS-17 and QS-18, or QS-7, QS-17, and QS-18 administered together. Purified saponins may also be administered together with non-saponin adjuvants. Such non-saponin adjuvants useful with the present invention are oil adjuvants (for example, Freund's Complete and Incomplete), liposomes, mineral salts (for example, $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, alum, $Al(OH)_3$, $Ca_3(PO_4)_2$, kaolin, and carbon), polynucleotides (for example, poly IC and poly AU acids), and certain natural substances (for example, wax D from *Mycobacterium tuberculosis*, as well as substances found in *Corynebacterium parvum*, *Bordetella pertussis*, and members of the genus *Brucella*), conjugates to the

carrier proteins, such as bovine serum albumin, diphtheria toxoid, tetanus toxoid, edestin, keyhole-limpet hemocyanin, Pseudomonal Toxin A, cholera-genoid, cholera toxin, pertussis toxin, viral proteins, and eukaryotic proteins such as interferons, interleukins, or tumor necrosis factor. Such proteins may be obtained from natural or recombinant sources according to methods known to those skilled in the art. When obtained from recombinant sources, the non-saponin adjuvant may comprise a protein fragment comprising at least the immunogenic portion of the molecule. Other known immunogenic macromolecules which may be used in the practice of the invention include, but are not limited to, polysaccharides, tRNA, nonmetabolizable synthetic polymers such as polyvinylamine, polymethacrylic acid polyvinylpyrrolidone, mixed polycondensates (with relatively high molecular weight) of 4'4'-diaminodiphenyl-methane-3,3'-dicarboxylic acid and 4-nitro-2-aminobenzoic acid (See Sela, M., *Science 166*:1365-1374 (1969)) or glycolipids, lipids or carbohydrates. A preferable adjuvant is alum, which gives a 3-fold increase in IgG1 response.

The saponins may also be directly linked to the antigen or may be linked *via* a linking group. By the term "linker group" is intended one or more bifunctional molecules which can be used to covalently couple the saponin or saponin mixture to the OspA and OspB proteins and which do not interfere with the production of antigen-specific antibodies *in vivo*. The linker group may be attached to any part of the saponin so long as the point of attachment does not interfere with the production of antigen-specific antibodies *in vivo* and thus interfere with the induction of active immunity.

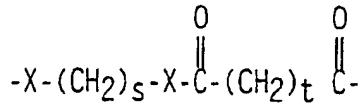
Examples of linker groups which can be used to link the saponin to the OspA and OspB proteins may comprise

$-NH-(CH_2)_q-NH-$,

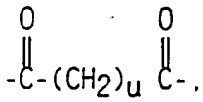
wherein q is 2-10;

$-O-(CH_2)_r-NH-$,

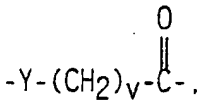
wherein r is 2-10;



wherein X = NH, S or O, s = 2-5, t = 2-12;



wherein u = 2-12;



wherein Y is NH or S, v = 1-3.

Typically, the saponins are linked to the OspA and OspB proteins by the preparation of an active ester of glucuronic acid, a component of the saponins, followed by reaction of the active ester with a nucleophilic functional group on the protein. Examples of the active esters which may be used in the practice of the invention include the glucuronate of N-hydroxysuccinimide, sulfo-N-hydroxysuccinimide, hydroxybenzotriazole, and p-nitrophenol. The active esters may be prepared by reaction of the carboxy group of the saponin with an alcohol in the presence of a dehydration agent such as dicyclohexylcarbodiimide (DCC), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDCI). The use of EDC to form conjugates is disclosed in U.S. Patent No. 4,526,714 to Feijen *et al.* and PCT application publication no.

WO91/01750, and Arnon, R. *et al.*, *Proc. Natl. Acad. Sci. (USA)* 77:6769-6772 (1980), the disclosures of which are fully incorporated by reference herein. The protein is then mixed with the activated ester in aqueous solution to give the conjugate.

Where a linker group between the saponin and the protein is desired, the active ester of the saponin glucuronate is prepared as described above and reacted with the linker group, e.g. 2-aminoethanol, an alkylene diamine, an amino acid such as glycine, or a carboxy-protected amino acid such as glycine *tert*-butyl ester. If the linker contains a protected carboxy group, the protecting group is removed and the active ester of the linker is prepared (as described above). The active ester is then reacted with the antigen to give the conjugate. Alternatively, the antigen may be derivatized with succinic anhydride to give an antigen-succinate conjugate which may be condensed in the presence of EDC or EDCI with a saponin-linker derivative having a free amino or hydroxyl group on the linker. See WO91/01750.

Once derivatized at the glucuronate carboxyl with a linker group, the saponins retain adjuvant activity. Those saponin derivatives prepared by reductive alkylation at the triterpene aldehyde do not appear to retain adjuvant activity at doses less than 40 μg. However, derivatives in which the saponin triterpene aldehyde was reduced to an alcohol by sodium borohydride reduction did retain some activity.

It is also possible to prepare a saponin conjugate comprising a linker with a free amino group (derived from an alkylene diamine) and crosslink the free amino group with a heterobifunctional cross linker such as sulfosuccinimidyl 4-(N-maleimidocyclohexane)-1-carboxylate which will react with the free sulfhydryl groups of protein antigens.

The saponin may also be coupled to a linker group by reaction of the aldehyde group of the quillaic acid residue with an amino linker to form an intermediate imine conjugate, followed by reduction with sodium borohydride or sodium cyanoborohydride. Examples of such linkers include amino alcohols such as 2-aminoethanol and diamino compounds such as

ethylenediamine, 1,2-propylenediamine, 1,5-pentanediamine, 1,6-hexanediamine, and the like. The antigen may then be coupled to the linker by first forming the succinated derivative with succinic anhydride followed by condensation with the saponin-linker conjugate with DCC, EDC or EDCI.

In addition, the saponin may be oxidized with periodate and the dialdehyde produced therefrom condensed with an amino alcohol or diamino compound listed above. The free hydroxyl or amino group on the linker may then be condensed with the succinate derivative of the protein in the presence of DCC, EDC or EDCI.

The ratio of saponin molecules per protein molecule may vary considerably according to the molecular weight of the antigen, the number of binding sites on the protein capable of being coupled to the saponin, and the antigenic characteristics of the particular saponin. In general, the ratio of saponin molecules to protein molecules may be about 0.1:1 to about 10:1. Preferably, the ratio may range from about 1:1 to about 3:1.

Administration of the vaccines of present invention may be by parenteral, intravenous, intramuscular, subcutaneous, intranasal, or any other suitable means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the antigen administered. In general, the vaccines may be administered at a dosage of about 0.01 to about 1.0 mg/kg of protein and saponin per weight of the individual. The initial dose may be followed up with a booster dosage after a period of about four weeks to enhance the immunogenic response. Further booster dosages may also be administered.

The effective compound useful in the method of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline, or phosphate-buffered saline, or any such carrier in which the compounds used in the method of the present invention have suitable solubility properties for use in the methods of the present invention.

The vaccines of the present invention may also be encapsulated within liposomes according to U.S. Patent No. 4,235,877 to Fullerton.

The invention also provides for a kit for the immunization of an animal comprising a carrier compartmentalized to receive in close confinement therein one or more container means wherein a first container contains the OspA and OpsB proteins and, optionally, the saponin. The kit may instead include at least one other container means which contain a saponin adjuvant or other adjuvant as described herein.

The invention also relates to a method of inducing immunity to *B. burgdorferi* in an animal, comprising administering to the animal a vaccine comprising OspA, OspB or fragments thereof; and a saponin adjuvant.

Having now generally described the invention, the same may be further understood by reference to the following examples, which are not intended to be limiting unless so expressly stated.

Example 1 ***A Colorimetric Microtiter Assay for Borreliacidal Activity of Antisera***

Materials and Methods

Bacterial Strains and Growth Conditions

Borrelia burgdorferi strains B31 (Burgdorfer, W., *et al.*, *Science 261*:1317-1319 (1982)) and Fr isolated from ticks in Germany were kindly provided by H.-J. Wellensieki, Klinikum Der Justus-Liebig-Universitat Giessen, Germany. Strains G25 (Postic, D., *et al.*, *Res. Microbiol. 141*:465-475 (1990)) and Cr, isolated from ticks in Sweden and Wisconsin, USA, were generously provided by A. Barbour, University of Texas Health Science Center, San Antonio, TX, and S. Callister, Gundersen Medical Foundation, La Crosse, WI, respectively. Spirochetes were grown in mBSK medium at 32°C as described previously (Callister, S.M., *et al.*, *J. Clin. Microbiol. 28*:363-365 (1990)).

Antisera and Normal Sera

Antiserum to lysates of *B. burgdorferi* was raised by subcutaneous immunization of C3H/HeJ mice at age of 6 to 7 weeks. One hundred μl of the spirochete lysates containing 25 μg of protein in saline was mixed with an equal volume of Freund's Complete Adjuvant for the first immunization. The same dose of the antigen was mixed with an equal volume of Freund's Incomplete Adjuvant, and given for the next two immunizations at two week intervals. Antiserum to a recombinant outer surface protein A derived from strain B31 (OspA-B31) (Hung, C.-H, *et al.*, "Expression, purification and characterization of outer surface protein A (OspA) and B (OspB) from the Lyme disease spirochete, *Borrelia burgdorferi*," *V Int. Conf. Lyme Borreliosis*, Arlington, VA, USA (1992)) was produced by subcutaneous immunization of the mice 3 times with 200 μl of the antigen containing 25 μg of the recombinant protein and 20 μg of saponin adjuvant (QS-21) (Kensil, C.R., *et al.*, *J. Immunol. 146*:431-437 (1991)) at two week intervals. Antiserum was isolated two weeks after the last immunization. Normal guinea pig complement (276 $C'H_{50}$/ml) (GIBCO, Grand Island, NY) was stored at -80°C. The complemented was filter-sterilized and used immediately after thawing.

Colorimetric Borreliacidal Assay

This CBA was performed in 96-well plate (Costar, Cambridge, MA). The appropriate concentration for each reagent used in the CBA was first determined by checkerboard titration. Each serum sample was prepared by mixing an equal volume of sera from at least ten mice, inactivated by heating at 56°C for 45 min and sterilized by filtration through a 0.45 μm centrifuge filter (Spin-X, Costar, Cambridge, MA). Each sample was tested in triplicate. The sera were serially diluted in 100 μl of mBSK containing 120 μg/ml phenol red (SIGMA, St. Louis, MO) (mBSK-PR). Five μl of guinea pig complement were added to each well, and mixed. The spirochete culture in mBSK at the logarithmic growth phase was centrifuged for 8 min at 9000 x g and

resuspended in fresh mBSK medium. Ninety-five μl of spirochete suspension (containing approximately 4 x 10^6 spirochetes) were added to each well (final volume 200 μl). After mixing, the plates were incubated at 32°C for 2 to 5 days. The absorbance at 562/630 nm of each well was measured using a microplate reader (Bio-tek Instruments, Burlington, VT) before and every 24 h after incubation. For some assays, 5 μl of mBSK were applied instead of guinea pig complement. Controls consisting of mBSK instead of serum with or without complement were also included in each assay. For evaluation of killing percentage, the absorbance at 562/630 nm of the bacterial growth controls containing different concentration of spirochetes in mBSK was also determined by incubation of 100 μl of mBSK-PR with 100 μl of the serially diluted spirochetes in mBSK at 32°C.

Radioactive [^{3}H]-Thymidine Incorporation Assay

The [^{3}H]-thymidine uptake by *B. burgdorferi* was performed in triplicate in 96-well plates. One hundred μl of borrelial suspension (approximately 8 x 10^6 spirochetes) was serially (two-fold) diluted in mBSK, and incubated with an equal volume of mBSK-PR at 32°C. After 30 h incubation, 20 μl of [^{3}H]-thymidine in mBSK (100 μCi/ml) were added into each well. After another 18 h of incubation for pulse-labeling the live spirochetes (Pavia, C.S., *et al.*, *J. Infect. Dis. 163*:656-659 (1991)), the absorbance at 562/630 of the plate was measured by a microplate reader, and the radioactivity was determined. Samples were harvested onto glass-fiber filter and washed 20 times using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). Each filter disk retaining spirochetes was transferred into a scintillation vial containing scintillation cocktail (CytoScint, ICN Biomedicals, Inc., Irvine, CA), and the radioactivity measured as counts per minutes using liquid scintillation system LS 1701 (Beckman Instruments, Inc., Irvine, CA).

Results

The present CBA is based upon color changes that occur resulting from the accumulation of nonvolatile acid produced by actively metabolizing spirochetes after incubation of a certain period of time in the presence of phenol red. The color changes due to the accumulation of the acid could be detected qualitatively by the naked eye or quantitatively by a microplate reader. A number of factors, such as concentration of phenol red, wavelength used for measuring the absorption, spirochete concentration, and buffering capacity of the growth medium could affect the CBA. Solutions containing different concentrations of phenol red (2.3 μg/ml to 133 μg/ml) were first titrated with HCl (final concentration of 0.77 mM to 9.6 mM), and the absorbance was determined using various single wavelengths (405 nm, 450 nm, 490 nm, 562 nm, 595 nm, and 630 nm) or dual wavelengths (405/630, 450/630, 490/562, 562/630). The concentration of phenol red (final concentration 60 μg/ml) and dual wavelength (562/630 nm) were chosen because they reflected the maximal absorbance change. A proper concentration of spirochetes used in this assay was also tested. With low concentrations of the spirochetes, the color or absorbance change of the assay mixture was delayed or minimized due to the inadequate accumulation of acids. While with high concentrations of spirochetes, more specific antibodies would be required to kill the bacteria and inhibit the color change. Thus, an optimal concentration of 4 x 10^6 spirochetes was chosen to ensure the sensitivity of this assay. A yellow color of the assay mixture after incubation for 48 to 72 h indicates growth of *Borrelia*, whereas a red color represents either no borrelial growth or borrelial death (Fig. 1).

To confirm the validity and reliability of the colorimetric assay, both [^{3}H]-thymidine uptake by live spirochetes and colorimetric changes due to borrelial growth were determined simultaneously with different concentrations of the bacteria. The [^{3}H]-thymidine incorporation by spirochetes or changes of the absorbance were dose-dependent (Fig. 2). The results of TIA

correlated well with those of the colorimetric assay (R=0.977), and were linearly related from an absorbance of 0.4 to 1.3 (Fig. 2).

The CBA of mouse antisera to OspA-B31 is shown in Fig. 3. At nearly all serum dilutions, the absorbance readings increased from the 0 to 24 h time points. However, by 48 h after incubation, the absorbance readings decreased, and continued to decrease in a regular fashion through 120 h at all serum concentrations tested. With each of the first three dilutions (1:10 to 1:40), the absorbance decreased approximately 0.1 optical density every 24 h. The absorbance decreased more dramatically as antisera were diluted further.

Mouse antisera to strain B31 lysates strongly inhibited the growth of the homologous strain B31 after 48 h, as indicated by the high absorbance. However, normal mouse sera did not affect the growth of spirochete (Fig. 4), and was similar to borrelial growth controls in mBSK.

The borreliacidal TIA and CBA using serially diluted antisera to B31 lysates were also done simultaneously. These two assays correlated well (R=0.935). The TIA and CBA also showed that the heat-inactivated mouse antisera in the presence or absence of guinea pig complement could equally and efficiently inhibit the growth of the spirochetes. Dark-field microscopy indicated that the number of live spirochetes were significantly reduced, and dead spirochetes were visible in the presence of the antisera, but not in the presence of the normal mouse sera.

Example 2

Immunization of Animals with OspA, OspB and QS-21

The genes encoding strain B31 OspA or OspB were cloned using the published DNA sequences (Bergstrom, S. *et al., Mol. Microbiol. 3*:479-486 (1989), the contents of which are fully incorporated by reference herein) and standard molecular biological techniques (Maniatis, T. *et al.*, Molecular Cloning. Cold Spring Harbor Laboratory, New York (1982). Recombinant

OspA starting at amino acid 17 and OspB starting at amino acid 20 were expressed in *E. coli* using a heat-inducible pL promotor (Mariciani, D.J. *Vaccine 9*:89-96 (1991); Nagi, K. *et al., Nature 309*:810-813 (1984)). In addition, each recombinant protein contained 16 amino acids (MVRABKRBEALRIAGS) (SEQ ID NO:1) at its N-terminus derived from a bacterial leader and nucleotide linker sequence. Expression of OspA and OspB was confirmed with specific monoclonal antibodies to OspA and OspB of strain B31 (Cambridge Biotech, Corp., Worcester, Mass). OspA and OspB were purified by reversible citraconylation and anion exchange chromatography as described previously (Marciani, D.J. *et al.*, in R. Burgess (ed.), protein purification, Alan R. Liss, Inc., New York, pp.443-458.; Marciani, D.J. *et al., Vaccine 9*:89-96 (1991)).

Antisera were prepared by subcutaneous immunization of 10 month old Beagles twice at two week interval with one ml of the Lyme vaccine. The vaccine contains 100 μg of truncated OspA and/or 100 μg of truncated OspB formulated with 50 μg of adjuvant QS-21. Antisera were isolated two weeks after second immunization. Borreliacidal activity of the sera was determined by CBA. Borrelial culture at logarithmic growth phase was centrifuged for 8 min at 9,000 xg at 15°C and resuspended in fresh mBSK. Ninety five μl of the borrelial suspension (containing approximately $4x10^6$ of spirochetes) and 5 μl of guinea pig complement were mixed with an equal volume of the serially diluted heat-inactivated antisera in mBSK containing 120 μg of phenol red in microtiter plate, and incubated for 72 h at 32°C. Absorbance at 562/630 was measured by a microtiter reader. The results are shown in Figure 5. High absorbance indicated borrelial death and high borreliacidal activity of the antisera. The low absorbance represented borrelial survival and growth. The error bars represent the standard error of 16 measurements of 8 serum samples for antisera and 8 measurements of 4 preimmune sera.

Figure 6 shows the borreliacidal activity of canine antisera to truncated OspA and/or OspB derived from *B. burgdorferi* strain B31 against the heterologous California strain CA-2-87. Sera preparation and borreliacidal

assay were described as above except that the incubation time of borreliacidal assay was 96 h. High absorbance indicated borrelial death and high borreliacidal activity of the antisera. The low absorbance represented borrelial survival and growth. The error bars represent the standard error of 16 measurements of 8 serum samples for antisera and 8 measurements of 4 preimmune sera.

Figure 7 shows the borreliacidal activity of individual canine antisera to truncated OspA and/or OspB derived from *B. burgdorferi* strain B31 against the homologous strain. Sera preparation and borreliacidal assay were described as above. This figure showed borreliacidal activity of individual dog antiserum and preimmune serum at 1:20 dilution. High absorbance indicated borrelial death and high borreliacidal activity of the antisera. The low absorbance represented borrelial survival and growth.

Figure 8 shows the borreliacidal activity of individual canine antisera to truncated OspA and/or OspB derived from *B. burgdorferi* strain B31 against the heterologous California strain CA-2-87. Sera preparation and borreliacidal assay were described above. The figure shows borreliacidal activity of individual dog antiserum and preimmune serum at 1:80 dilution. High absorbance indicated borrelial death and high borreliacidal activity of the antisera. The low absorbance represented borrelial survival and growth.

Figure 9 shows the borreliacidal activity of C3H/HeJ female mice that had been immunized twice with either 25 μg of truncated OspA, 25 μg of truncated OspB, or 25 μg of truncated OspA and 25 μg of truncated OspB. Sera from ten mice in each group were pooled and tested for borreliacidal activity against the homologous strain B31 or the highly divergent Swedish strain G25, as described above.

Figure 10 shows the borreliacidal activity of C3H/Hej female mice that had been immunized twice with either 25 μg of truncated OspA + 20 μg of QS21, 25 μg of truncated OspB + 20 μg of QS21, or 25 μ of truncated OspA and 25 μg of truncated OspB + 20 μg of QS21. Sera from ten mice in each group were pooled and tested for borreliacidal activity against the homologous

strain B31 or the highly divergent Swedish strain G25, as described above. Surprisingly, in dogs but not in mice, the results show that a combination of OspA and OpsB, when administered together with QS-21, results in a much greater borreliacidal activity when compared to when OspA was administered alone (Figures 5-9).

Example 3 ***Characterization of Canine humoral Immune Responses to Osp Subunit Vaccines and to Natural Infection by Lyme Disease Spirochetes***

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains were described above, in the corresponding section of Example 1 of the present specification. *Borrelia burgdorferi sensu stricto* strains B31, CA-2-87, and *B. garinii sp. nov.* strain G25 were also described elsewhere (Baranton *et al., Int. J. Sys. Bacteriol. 42*:378-83 (1992); Ma *et al., J. Microbiol. Methods 17*:145-53 (1993); Marconi *et al., Infect. Immun. 61*:2611-7 (1993)), the entire texts of which are herein incorporated by reference. Strain 24008 Fr, a French isolate, was generously provided by David Dennis (Center for Disease Control, Collins, CO). Spirochetes were grown in modified Barbour-Stoenner-Kelly (mBSK) medium at 32°C as previously described (Callister, S. M. *et al., J. Clin. Microbiol. 28*:363-386 (1990)), the entire text of which is herein incorporated by reference.

More specifically, *E. coli*, strain MZ-1 was used as the host strain for expression of recombinant antigens. This strain was a gift from Takis Papas (National Cancer Institute, December 1984) and has the following genotype: $galK_{am}$ Δ $BamN_7M_{53}CI857$ Δ H1, his^-, ilv^-, bio^-, N^+. (Nagai & Thogersen, *Nature 309*:810 (1984)). The expression vector was used to transfect MZ-1 strain cells with replication deficient bacteriophage lambda DNA sequences containing a temperature sensitive *CI857* transcription repressor gene. Under

permissive temperature conditions, 32°C, this repressor protein is active, inhibiting the synthesis of recombinant proteins. By shifting the cell culture to a nonpermissive temperature, 42°C, protein synthesis is induced. Use of this temperature sensitive mutant allows dense cell cultures to be achieved prior to the synthesis of recombinant proteins which may be toxic to the *E. coli*. The plasmids for expression of OspA and OspB were each transfected into host cell line MZ-1 which were then used for induction of recombinant antigens.

Production of OspA and OspB

OspA and OspB were produced according to Example 4 of the present specification and as further described below. The genes encoding OspA and OspB of *B. burgdorferi sensu stricto* strain B31 were cloned using published DNA sequence (Bergstrom, S. *et al.*, *Mol. Microbiol. 3*:479-486 (1989)) and standard molecular biological techniques (Maniatis, T. *et al.*, *Molecular Cloning*, Cold Spring Harbor Laboratory, New York (1982)). Recombinant OspA starting at amino acid 17 and OspB starting at amino acid 20 were expressed in *E. coli* strain MZ-1 using expression vector pLCBC1 (Beltz, G. A. *et al.*, U.S. Patent No. 4,753,873 (1988)), a vector very similar to that described by Lautenberger (Lautenberger, J. A. *et al.*, *Gene Anal. Techn. 1*:63-66 (1984)) and used to express recombinant proteins to high level (Lautenberger, J. A. *et al.*, *Gene 23*:75-84 (1983)). The vector makes use of the heat inducible pL promoter; and Shine-Delgarno sequence and first N-terminal 16 amino acid codons are derived from the Lambda cII gene. Cloning sites within three codons of mature OspA and OspB were chosen for ligation into the expression vector. The cloned OspA and OspB genes were confirmed by DNA sequencing (Bergstrom, S. *et al.*, *Mol. Microbiol. 3*:479-486 (1989)). Expression of OspA and OspB was confirmed with specific monoclonal antibodies to OspA and OspB of strain B31 and polyclonal antiserum to the lysates of strain B31 (Ma, J. *et al.*, *Program and abstracts of V Int. Conf. Lyme Borreliosis*, Arlington, Virginia (1992)). OspA and

OspB were purified by reversible citraconylation and/or anion exchange hromatography as described previously (Marciani, D. J. *et al.*, *Vaccine 9*:89-96 (1991); Marciani, D. J. *et al.*, in R. Burgess, ed., *Protein Purification*, Alan R. Lise, Inc., New York (1987) pp. 443-458). The truncated OspA and OspB started at amino acid 17 and 20, respectively. Recombinant proteins were expressed in *Escherichia coli*, confirmed, and purified as previously described (Marconi *et al.*, *Infect. Immun. 61*:2611-7 (1993)), the entire text of which is herein incorporated by reference.

More specifically, the expression plasmids pCBC1OspA8+6 and pCBC1OspB8+4, used to produce full length lipidated OspA and OspB proteins, were constructed and tested as follows:

A. OspA and OspB Coding Sequences

B. burgdorferi strain B31 (Burgdorfer *et al.*, *Science 261*:1317-1319 (1982)) was received from H.J. Wellenski, Klinikum Der Justus-Liebig-Universitat Giessen, Giessen, Germany. Total DNA extracted from this strain was used to subclone the OspA and OspB protein encoding regions separately. Figure 21 details each protein encoding region as they occur in *B. burgdorferi* strain B31.

B. Expression Vector Construction

The expression vector used to express *B. burgdorferi* recombinant OspA and OspB antigens was constructed by Cambridge Biotech Corporation (CBC) scientists and is based on published work (Shimatake & Rosenberg, *Nature 291*:128 (1981); Lautenberger *et al.*, *Gene 23*:75 (1983); Lautenberger *et al.*, *Gene Analysis Techniques 1*:63 (1984)). The vector uses the bacteriophage lambda pL promoter, and a fragment of the lambda c11 gene which provides a ribosome binding site, ATG initiation codon, and 12 additional codons. A BamHI site follows which is used for cloning DNA fragments to be expressed, which in turn is followed by a polytranslation terminator. The basic features are presented in Figure 22. Commercially obtained lambda and pBR322 DNA were used as starting materials.

C. Cloning of OspA and OspB for Expression

1. The OspA BamHI-digested fragment was subcloned into The Cambridge Biotech expression vector, pLCBC1, also restricted with BamHI. Plasmids from colonies that contained the 907 base pair insert were also digested with EcoRI (located distally to the 5′ end) to determine the orientation of the fragment within pLCBC1. The correctly oriented clone was named pLCBC1OspA8+6. Figure 23 details the entire sequence of pLCBC1OspA8+6 and the origin of each base.

2. The OspB BamHI-restricted fragment was also subcloned (separately) into pLCBC1 restricted with BamHI. Plasmids from colonies that contained the 945 base pair fragment when digested with BamHI were also digested with HindIII to determine the direction of the insertion. The clone with the correct orientation was named pLCBC1OspB8+4. Figure 24 details the entire sequence of pLCBC1OspB8+4 and shows the origin of each base.

Table 4. Validation of the OspA Clone

A series of restriction enzyme digests confirming pLCBC1OspA8+6 were performed. The size of the DNA fragments expected is also presented.

RESTRICTION MAPPING OF PLCB1OSPA8+6 (OSPA)

Restriction Digests	Predicted Fragment Sizes (bp)
BamHI	3852, 902
PvuII	4754
EcoRI	3354, 1400
HindIII	4754
NdeI	2766, 1988
PstI	2612, 2142
ScaI	3265, 1489
EcoRI + PvuII	3354, 880, 520
EcoRI + HindIII	3354, 930, 470
PstI + PvuII	2612, 1268, 874

The size of restriction fragments observed on the gel were indistinguishable from those predicted. See Figure 25. A deposit of the expression vector pLCBC1OspA8+6 was made in compliance with the provisions of the Budapest Treaty, at American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA, on February 25, 1994, under accession number 69577.

Table 5. Validation of the OspB Clone

A series of restriction enzyme digests confirming pLCBC1OspB8+4 were performed. The size of the DNA fragments expected is also presented.

RESTRICTION MAPPING OF PLCBC1OSPB8+4 (OSPB)

Restriction Digests	Predicted Fragment Sizes (bp)
BamHI	3852, 900
EcoRI	4752
NdeI	2766, 1986
PstI	2562, 2190
PvuII	3700, 1052
BamHI + EcoRI	3105, 900, 747
EcoRI + HindIII	3429, 1323
EcoRI + PstI	2562, 1442, 748
HindIII + SspI	3240, 1512
PvuII + SspI	2991, 1052, 709

The size of restriction fragments observed on the gel were indistinguishable from those predicted. See Figure 26. A deposit of the expression vector pLCBC1OspB8+4 was made in compliance with the provisions of the Budapest Treaty, at American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA, on February 25, 1994, under accession number 69578.

Monoclonal antibody

Murine monoclonal antibody (MAb) to OspA and OspB of *B. burgdorferi sensu stricto* strain B31 were produced with modification of previously described procedures (Lane, R.D., *J. Immunol. Methods 81*:223-8 (1985); Kennett, R.H., "Hybridomas: A New Dimension in Biological Analysis," in *Monoclonal Antibodies*, Kennett *et al.*, Plenum Publ. Corp., New York (1980), pp. 365-7). Briefly, Balb/c mice were immunized at least two times with recombinant OspA, OspB, or borrelial lysate. Primed spleen cells were isolated and fused with the myeloma cell line SP2/0 by mixing at a 4:1 ratio in the presence of 50% polyethyleneglycol 1500 (Boehringer Mannheim, Indianapolis, IN). Hybridoma cultures producing antibodies of interest were cloned at least twice by limiting dilution. MAbs to OspA and OspB were identified by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Antibody isotypes were determined by ELISA using isotype-specific goat anti-mouse IgG conjugated to horseradish peroxidase (Pel-Freez, Rogers, AZ). MAbs were purified from culture supernatant using protein-A affinity column, and protein concentration determined by BCA assay (Pierce, Rockford, IL).

Intrinsic radiolabeling of OspA and OspB and immunoprecipitation

Escherichia coli strain MZ-1 harboring *ospA* or *ospB* gene was grown in 5 ml of LB broth to log phase at 32°C. Twenty μl of [9,10-^{3}H]palmitic acid (52.4 Ci/mmol, Dupont Nen, Boston, MA) were added to the culture and incubated for another 2 h at 42°C for heat induction of recombinant proteins. Bacteria were washed twice with cold sterile water, lysed with the solution of 0.05 Tris-HCl (pH 6.8), 1% Triton X-100, 0.5% sodium deoxycholate, 0.15% SDS, 0.15 M NaCl (Katona *et al., Infect. Immun. 60*:4995-5003 (1992)), and boiled for 10 minutes. The supernatant was incubated with mixture of purified OspA specific MAbs H3G4, L3B5, T1F6, and T2H12, and with OspB specific MAb P4D1, respectively, for 2 h at 22°C with gentle agitation, and then incubated with protein G-Sepharose 4FF (Pharmacia,

Piscataway, NJ) in 0.25 M Tris-HCl, pH 6.8, for another 1 h at 22°C. The mixture was microcentrifuged and washed 3 times, and resuspended in distilled water. The isolated recombinant proteins were mixed with sample buffer (Laemmli, U.K., *Nature (London) 227*:680-5 (1970)), boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Vaccine formulation

Vaccine formulation was prepared as described in Example 4, below; also *see* Kensil *et al., J. Immunol. 146*:431-7 (1990). Each dose (1 ml) of vaccine contained 100 or 25 μg each of FLOspA and/or FLOspB formulated with or without 50 μg of QS-21 (Kensil *et al., J. Immunol. 146*:431-7 (1990), in saline, pH 6.5. A vaccine containing 100 μg each of TOspA, TOspB and 50 μg of QS-21 was also prepared for comparative study.

Vaccination and antiserum

Beagle dogs (Harlan Sprague Dawley, Indianapolis, IN) at age of 12 weeks were vaccinated subcutaneously with 1 ml of vaccine. Immune response was boosted once 4 weeks later with the same dose of vaccine. Antiserum was isolated 2 weeks after the second vaccination and stored at -20°C.

Naturally exposed canine sera

Serum samples isolated from naturally exposed dogs in Westchester County and Long Island, New York, a highly endemic area for Lyme disease (Alpert *et al., NY State J. Med. 92*:5-8 (1992)) were kindly provided by H. Schneider, Vet Research, Farming Dale, New York, and Durland Fish, New York Medical College, Valhalla, New York. Those serum donors were not vaccinated with Ft. Dodge Lyme disease bacterin. Normal dog sera used as negative control were isolated from dogs raised in closed kennels (Harlan Sprague Dawley, Indianapolis, IN).

SDS-PAGE and immunoblotting

SDS-PAGE was performed using a 11% separating gel (Edelman, R., *Vaccine 9*:531-2 (1991)). Immunoblotting was conducted by modification of the procedure of Towbin *et al.* (Towbin *et al., Proc. Natl. Acad. Sci. USA* 76:4350-4 (1979)). For testing canine serum samples, sera were diluted at 1:50, and immunoblot strips were used according to the instruction of manufacturer (Cambridge Biotech Corporation, Worcester, MA). The goat anti-dog IgG $F(ab')_2$ conjugated onto horseradish peroxidase (Bethyl Laboratories, Inc., Montgomery, TX) were used to detect the specific antibody in serum samples.

ELISA

ELISA was performed using plates coated with *B. burgdorferi* antigens, 0.2 μg OspA per well , and 0.2 μg OspB per well, respectively, as described (Lindenmayer *et al., J.Clin. Microbiol. 28*:92-6 (1990)). Antibody isotypes were determined using isotype-specific goat anti-dog IgG conjugated to horseradish peroxidase (Bethyl Laboratories, Inc., Montgomery, TX).

Antiborrelial assay

A colorimetric microtiter assay for antiborrelial activity of antisera was performed as described above. Briefly, heat-inactivated, filter-sterilized sera were serially diluted in 100 μl of mBSK containing 120 μg of phenol red per ml in 96-well plates. Five μl of sterile guinea pig complement (Gibco BRL, Gaithersburg, MD) and 95 μl of spirochete (containing approximately 6×10^7 of spirochetes) in fresh mBSK were added to each well. After 48 to 72 h incubation at 32°C, the absorbance at 562/630 nm was measured using a microplate reader. Antiborrelial activity was expressed by either absorbance change compared with those of preimmune sera or by titer. The titer of antiborrelial activity was defined as the highest dilution of the antisera which inhibited absorbance change caused by 50% of spirochetes used in this assay.

Antiborrelial activity reflects borrelial killing and borrelial growth inhibition by specific antibodies.

Statistical analysis

One-tailed Student's t-test was conducted to compare antibody titers of various groups of immune sera. Significance level was assigned assuming equal variances about the means using Microsoft Excel 4.0 for Macintosh.

Results

MAbs to OspA and OspB

A number of MAbs reactive with OspA and OspB of *B. burgdorferi sensu stricto* strain B31 were developed. MAbs H3G11, L3B5, T1F6, T2H12 (all IgG1) are specific for OspA, and MAb P4D1 (IgG1) is specific for OspB, as tested by ELISA and immunoblotting.

Radioimmunoprecipitation of OspA and OspB

MAb P4D1 specific for OspB precipitated OspB from lysate of *E. coli* labeled with [9,10-^{3}H]palmitic acid. A mixture of 4 OspA specific MAbs described above were used for precipitation of OspA. Lipidation of the precipitated OspA and OspB was analyzed by SDS-PAGE and fluorography. both TOspA and TOspB are nonlipidated proteins, and FLOspA and FLOspB are lipoproteins as shown by the radiolabeled bands (Fig. 11). FLOspB had another radiolabeled band with a molecular mass of approximately 22 KDa, perhaps a recombinant fragment from OspB (*see* Example 4, below).

Antibody responses to experimental Osp vaccines

Various experimental vaccines were evaluated in dogs for eliciting IgG isotype antibody responses (Fig. 12). A QS-21 formulated vaccine containing 100 μg each of FLOspA and FLOspB induced 30-fold higher ($p < 0.005$) IgG1 and 4-fold higher ($p < 0.0005$) IgG2 antibody responses than the same

formulation containing TOspA and TOspB. A vaccine containing 25 μg each of FLOspA and FLOspB was similar to the vaccine containing 100 μg each of the lipoproteins in terms of eliciting antibody titer. QS-21 enhanced at least 4-fold higher ($p<0.1$) IgG1 and 8-fold higher ($p<0.05$) IgG2 antibody responses when comparing FLOspA and FLOspB based vaccines formulated with and without QS-21. Either FLOspA and FLOspB based vaccine formulated with QS-21 elicited antibody responses similar to both FLOspA and FLOspB based vaccine formulated with QS-21 ($p<0.2$ and $p<0.1$, respectively).

All antisera to vaccines formulated with nonlipidated or lipoproteins OspA, OspB, and QS-21 contained antibodies to both OspA and OspB. The representative immunoblot showed that all 11 antisera to vaccine containing 25 μg each of FLOspA and FLOspB and 50 μg of QS-21 reacted with both OspA and OspB protein bands (Fig. 13, lane 13 to 23).

OspA and OspB specific antibodies in naturally exposed canine sera

Sixty three serum samples from naturally exposed dogs were tested positive for antibodies to *B. burgdorferi* by ELISA and immunoblotting. The representative patterns of immunoblot with these positive sera were shown in Fig. 13 (lanes 2 to 12). Among these positive sera (Table 1), 24 (38%) sera reacted with both OspA and OspB (Fig. 13, lane 2-7); 3 (5%) sera reacted with OspA, not OspB (lane 8) and 4 (6%) sera recognized OspB, not OspA (Lane 9-10). Most of OspA and OspB bands were faint. Thirty-two positive sera (51%) reacted with neither OspA nor OspB (Fig. 13, lane 11-12). OspA and OspB specific IgG1 and IgG2 antibody titers of these positive sera were low (Fig. 14). Most of them had antibody titers of $\leq$ 100. Only one serum sample had high titer of IgG1 and IgG2. In contrast, antisera elicited by the experimental vaccine containing 25 μg each of FLOspA, FLOspB and QS-21 had significantly ($p<0.001$) higher titer than naturally exposed dogs sera. (Fig. 14).

Antiborrelial activity of canine sera

The antiborrelial activity of the canine antisera to the experimental Lyme disease vaccines was tested against the homologous strain B31 and heterologous strain CA-2-87, as well as the other *Borrelial* species G25 and 24008 Fr. Antisera to QS-21 formulated vaccine containing TOspA and TOspB were antiborrelial to the homologous strain B31 and heterologous strain CA-2-87. Their antiborrelial titers, however, were 8-fold ($p<0.005$) and 3-fold ($p<0.001$) lower against B31 and CA-2-87, respectively, than those of antisera to the same formulation containing FLOspA and FLOspB (Fig. 15). Antisera to vaccine containing 25 μg each of FLOspA, FLOspB, and QS-21 possessed similar antiborrelial activity to those sera induced by vaccine containing 100 μg each of FLOspA, FLOspB, and QS-21 (Fig. 15). These antisera had significantly higher antiborrelial activity against homologous and heterologous Lyme disease spirochetes than antisera to the vaccines formulated without QS-21 or formulated with only either FLOspA or FLOspB, as indicated by higher absorbance (Fig. 16). Only these antisera to FLOspA, FLOspB, and QS-21 were also antiborrelial to *B. garinii sp. nov.* strain G25 and borrelial strain 24008 Fr, both European isolates (Fig. 16). On the basis of antiborrelial activity against strain B31, antiborrelial activity of the antisera correlated with isotype IgG2 antibody titer ($R=0.61$) better than with IgG1 antibody titer ($R=0.13$) (Fig. 17).

Among 63 naturally exposed, seropositive dog sera, 13 (21%) had antiborrelial activity against strain B31 (titer 10 to 160) (Table 1). Six of these 13 samples had antibodies to both OspA and OspB, and 7 samples had antibodies neither to OspA nor to OspB, as determined by immunoblotting.

Example 4 ***Impact of the Saponin Adjuvant QS-21 and Aluminum Hydroxide on the Immunogenicity of Recombinant OspA and OspB of Borrelia burgdorferi***

Materials and Methods

Bacterial strains and growth conditions

Borrelia burgdorferi strains B31, CA-2-87, Fr, and G25 are described in Example 1 and in the literature (*see* Schwan *et al., J. Clin. Microbiol. 26*:557-558 (1988)). Spirochetes were grown in modified Barbour-Stoenner-Kelly (mBSK) medium at 32°C as described in the literature (*see* Callister *et al., J. Clin. Microbiol. 28*:363-365 (1990)).

Molecular cloning, expression, and purification of OspA and OspB

The genes encoding strain B31 OspA and OspB were cloned using published DNA sequence (Bergstrom, S. *et al., Mol. Microbiol. 3*:479-486 (1989)) and standard molecular biological techniques (Maniatis, T. *et al., Molecular Cloning*, Cold Spring Harbor Laboratory, New York (1982)). Recombinant OspA starting at amino acid 17 and OspB starting at amino acid 20 were expressed in *E. coli* strain MZ-1 using expression vector pLCBC1 (Beltz, G. A. *et al.*, U.S. Patent No. 4,753,873 (1988)), a vector very similar to that described by Lautenberger (Lautenberger, J. A. *et al., Gene Anal. Techn. 1*:63-66 (1984)) and used to express recombinant proteins to high level (Lautenberger, J. A. *et al., Gene 23*:75-84 (1983)). The vector makes use of the heat inducible pL promoter; the Shine-Dalgarno sequence and first N-terminal 16 amino acid codons are derived from the Lambda cII gene. Cloning sites within three codons of mature OspA and OspB were chosen for ligation into the expression vector. The cloned *ospA* and *ospB* genes were confirmed by DNA sequencing (Bergstrom, S. *et al., Mol. Microbiol. 3*:479-486 (1989)). Expression of OspA and OspB was confirmed with specific monoclonal antibodies to OspA and OspB of strain B31 and polyclonal

antiserum to the lysates of strain B31 as described in Examples 1 and 2 (also *see* Ma and Coughlin, *J. Microbiol. Methods 17*:145-153 (1993)). OspA and OspB were purified by reversible citraconylation and/or anion exchange chromatography as described previously (Marciani D. J. *et al.*, *Vaccine 9*:89-96 (1991); Marciani, D. J. *et al.*, "Solubilization of inclusion body proteins by reversible N-acylation," in *Protein Purification*, R. Burgess, ed., Alan R. Liss, Inc., New York (1987), pp. 443-458).

Whole Cell Lysates

Borrelia burgdorferi culture at late logarithmic growth phase was centrifuged for 10 min at 10,000 xg, and the pellet washed 3 times with sterile 0.1 M phosphate-buffered saline, pH 7.2 (PBS). The pellet was resuspended in PBS containing 0.5% sodium dodecyl sulfate (SDS) and designated as whole cell lysates for immunoblotting analysis. Protein concentration of the lysates was determined by BCA assay (Pierce, Rockford, IL).

Adjuvants, Vaccine Formulations, and Antisera

QS-21 was purified from the cortex of the tree *Quillaja saponaria* monlina by ultrafiltration, adsorption chromatography, and reverse phase HPLC (Kensil, C. R. *et al.*, *J. Immunol. 146*:431-437 (1990)). For mouse antiserum production, each dose (0.2 ml) of the experimental vaccine contained 25 μg of recombinant protein. This antigen dose used was found to induce significantly higher titers of antibody than either 1 μg or 5 μg of antigen (data not shown). Formulated vaccines contained either 20 μg of QS-21 or 200 μg of alum (Alhydrogel, Accurate Chemical and Scientific Corp., Westbury, NY) in PBS, pH 6.5. The adjuvant doses of both QS-21 and alum were previously optimized for the water soluble protein antigen, ovalbumin in mice (Kensil, C. R. *et al.*, "The use of Stimulon™ to boost vacine response," *Vaccine Res.* 2:273-281 (1993).) For immunoprotection studies, the vaccine contained 25 μg each of OspA and OspB formulated with 20 μg of QS-21. Antisera to OspA and OspB were prepared by subcutaneous

immunization of 6 to 7 week old female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) 3 times at 2 week interval as described in Example 1 (also *see* Fikrig, E. *et al.*, *Science 250*:553-556 (1990)..

SDS-polyacrylamide Gel Electrophoresis and Immunoblotting

SDS- polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an 11% separating gel (Laemmli, U. K., *Nature (London) 227*:680-685 (1970)). The gel was stained with Coomassie Brilliant blue R-250. Immunoblotting was conducted by modification of the procedure of Towbin *et al.* (Towbin, H. *et al.*, *Proc. Natl. Acad. Sci. USA 76*:4350-5354 (1979)). Bacterial lysates (4 to 6 μg) were subjected to SDS-PAGE, transferred onto nitrocellulose membrane, and probed with both 1:20 and 1:200 dilutions of mouse antisera. Goat anti-mouse IgG conjugated to horseradish peroxidase (Fisher Scientific, Pittsburgh, PA) and 4-chloro-1-naphthol substrate was used to detect antigen-antibody binding on immunoblots.

Enzyme-linked Immunosorbent Assay

Appropriate concentration of each of the reagents used in the enzyme-linked immunosorbant assay (ELISA) was determined by checkerboard titration. The assay was performed using plates coated with *B. burgdorferi* antigens (Cambridge Biotech Corp., Worcester, MA) as previously described (Lindenmayer, J. *et al.*, *J. Clin. Microbiol. 8*:92-96 (1990)). For determination of antibody isotypes, an appropriate dilution of isotype-specific goat anti-mouse IgG conjugated to horseradish peroxidase (Fisher Scientific, Pittsburgh, PA) was used. Antibody titer was defined as the highest serum dilutions resulting in an absorbance value close to 1.0 in this assay.

Microaggultination

Borrelia burgdorferi culture was adjusted to a concentration which contained approximately 60 organisms per microscopic field. One hundred μl of *B. burgdorferi* was incubated in 96-well plates with an equal volume of

heat-inactivated, serially diluted antisera or normal serum in mBSK medium at 32°C for 2 hours. Agglutination was determined by dark-field microscopy. For each assay, 3 samples (10 μl) were taken and 9 fields of each sample were examined for individual spirochetes. The number of individual spirochetes per microscopic field was averaged. Agglutination titer was defined as the highest dilution of the antisera that produced less than 15 individual spirochetes per microscopic field (i.e. caused $\geq$ 50% of the spirochetes to agglutinate).

Colorimetric borreliacidal Assay

The colorimetric borreliacidal assay was performed in duplicate in 96-well plates (Costar, Cambridge, MA) by the method of the present invention and as described in Example 1 of the present specification. Briefly, heat-inactivated, filter-sterilized test sera were serially diluted in 100 μl of mBSK containing 120 μg/ml of phenol red (SIGMA, St. Louis, Missouri) (mBSK-PR). Five μl of sterile guinea pig complement (276 $C'H_{50}$/ml) (GIBCO, Grand Island, New York) and 95 μl of spirochete suspension (containing approximately 4 x 10^6 of spirochetes at logarithmic growth phase) in fresh mBSK were added to each well. After 48 to 72 h incubation at 32°C, the absorbance at 562/630 nm was measured using a microplate reader. The titer of borreliacidal activity was defined as the highest dilution of the antisera which inhibited absorbance change caused by 50% (2 x 10^6 bacteria) of spirochetes used in the assay. For each assay, positive and negative sera were also tested for controls.

Immunoprotection Studies

Groups of 10 C3H/HeJ mice were subcutaneously immunized two or three times with the experimental vaccine containing OspA and OspB formulated with QS-21. Control mice were injected with sterile 0.15 M saline. Two weeks after last immunization, mice were challenged intradermally with 10^3, 10^4, and 10^5 infectious spirochetes of strains B31 or

CA-2-87, respectively. In a separate experiment, all non-vaccinated mice were infected with as few as 10 spirochetes of either strain. Mice were sacrificed two weeks following the challenge, and the bladder, heart and blood samples were cultured for spirochetes as described (Schwan, T. G. *et al.*, *J. Clin. Microbiol.* *26*:557-558 (1988)). Heparinized blood and the organ extracts, each in a volume of 0.5 ml, were added to separate tubes containing 6 ml of BSK medium and incubated at 32°C. cultures were monitored for the presence of motile spirochetes every 2-3 weeks, using an acridine orange staining procedure and fluorescence microscopy as previously described (Pavia, C. S. *et al.*, *J. Infect. Dis.* *163*:656-659 (1991)).

Statistical Analysis

Two-tailed Student's t-tests were performed to compare antibody response to various groups. Significant levels were assigned assuming equal variance about the means using Microsoft Excel 4.0 for Macintosh.

Results

Production of Recombinant OspA and OspB

Expression of OspA and OspB in *E. coli* was induced by a shift of incubation temperature from 32°C to 42°C (lane 2 and 4, respectively, Fig. 18). Recombinant OspA and OspB were purified from *E. coli* (lane 3 and 5, respectively, Fig. 18), and confirmed by immunoblotting using monoclonal antibodies to OspA and OspB of strain B31. A few faint bands with low molecular weights in lanes 3 and 5 also reacted with the monoclonal antibodies on immunoblotting (data not shown), and are therefore not *E. coli* contaminants. Neither OspA and OspB were acylated (data not shown).

Electrophoretic and Antigenic Analysis

Different geographic isolates of *B. burgdorferi* were electrophoretically analyzed for protein composition. All 4 strains tested were similar to each other in the electrophoretic profile of the proteins except OspA and OspB (Fig. 19A). Strains B31 and Fr produced an approximately 31 Kda OspA and 34 Kda OspB (lane 1 and 3, Fig. 19A), respectively. Whereas, strain CA-2-87 produced an approximately 31 KDa OspA and 34.5 KDa OspB (lane 2, Fig. 19A); strain G25 produced an approximately 32 KDa OspA and 33.5 KDa OspB (lane 4, Fig. 19A). Immunoblotting of whole cell lysates of these 4 strains with antisera to OspA and OspB of strain B31 revealed that strains B31, CA-2-87, Fr, and G25 produced cross-reactive OspA and OspB. Antisera to OspB also reacted with an approximately 22 KDa protein band of strains B31, CA-2-87, and Fr (Fig. 19C).

Effect of Adjuvants on Antibody Responses to OspA and OspB

Antisera from mice immunized with OspA and OspB alone or formulated with either adjuvant QS-21 or alum were assayed for antibody responses. Isotypes IgM, IgG1, IgG2a, IgG2b, and IgG3 were measured by ELISA. Both OspA and OspB formulated without an adjuvant induced low titers of antibody response with the major isotype being IgG1 (Fig. 20). Titers of antibody response to OspA alone were significantly higher than those to OspB. QS-21 significantly enhanced the antibody responses of IgG2a and IgG2b to OspA ($p << 0.0001$ for both) and OspB ($p < 0.002$ and 0.02, respectively) (Fig. 20). QS-21 also significantly augmented the IgG1 antibody response to OspA ($p < 0.01$), but not to OspB. Alum had no significant effect on IgG2a and IgG2b responses to OspB ($p > 0.2$), but significantly inhibited IgG2a and IgG2b antibody responses to OspA ($p < 0.05$ and $p < 0.002$, respectively) (Fig. 20, part A and part C). The alum formulated vaccines enhanced IgG1 responses to OspB ($p < 0.01$) but not to OspA. Vaccines formulated with both QS-21 and alum augmented the antibody responses over those induced by alum alone, but less than QS-21 alone. IgM and IgG3

antibody responses to OspA or OspB formulated with either QS-21 or alum were minimal when compared with other IgG isotype antibody responses. As a result, it was not possible to detect an adjuvant effect on either IgM or IgG3 induction.

Microagglutination

Antisera to OspA formulated with QS-21 agglutinated not only the homologous New York strain B31, but also the heterologous California isolate CA-2-87 and German isolate Fr with agglutination titers of 1600 to 6400 (Table 1). This antisera also agglutinated the Swedish isolate G25 with a titer of 400. Antisera from mice vaccinated with OspA formulated with alum or without an adjuvant had agglutination titers of $\leq$ 50 to 1600. Antisera to OspB formulated with alum or QS-21 or without an adjuvant had much lower agglutination titers ($\leq$ 50 to 400) with the strains tested.

Borreliacidal Activity of Antisera

The representative results of the borreliacidal activity of antisera to OspA and OspB are shown in Table 2. Antisera to OspA formulated without an adjuvant had low borreliacidal activity against strains B31, Fr, and G25 (titers of 40 to 80), and had undetectable activity against strain CA-2-87 (titer of $\leq$ 10). QS-21 enhanced the borreliacidal anti-OspA response 8- to 64-fold as tested against these 4 strains (titers of 320 to 1280). Alum increased this response by less than 2-fold against the strains tested.

OspB formulated with either QS-21 or alum induced a 16-fold increase in borreliacidal activity against strain B31 when compared with OspB alone. A 16 fold increase in borreliacidal activity against strain Fr was also seen with antisera to OspB when formulated with QS-21, but not when formulated with alum. Neither of the antisera had borreliacidal activity against strain CA-2-87 or G25.

Immunoprotection

Vaccinated mice were protected from infection following challenge with as many as 10^5 infectious spirochetes (at least 4 log greater than the infectious dose) of either the strains B31 or CA-2-87. All vaccinated mice were free of spirochetes in bladder, heart, and blood. In contrast, all control mice were infected after challenge as shown by culturing spirochete from bladder tissue samples. Some control mice also had culture positive blood and heart tissue.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below. All patents and publications cited herein are fully incorporated by reference herein in their entirety.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 49, line 6

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
25 February 1994	69577

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

PLCBC10spA8+6

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 49, line 27.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
25 February 1994	69578

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

pLCBC10spB8+4

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
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Authorized officer ELNORA Y. RIVERA INTERNATIONAL DIVISION	Authorized officer

Form PCT/RO/134 (July 1992)

What Is Claimed Is:

1. A method for detecting the ability of an antiserum to *B. burgdoferi* to kill or inhibit the growth of *B. burgdorferi*, which comprises

(a) contacting said antiserum with *B. burgdorferi* and a suitable color pH indicator for measurement of bacterial growth in a medium which is compatible for the growth of *B. burgdorferi* in the absence of said antiserum; and

(b) measuring the absorbance of the sample;

wherein high absorbance, when compared to a control sample which does not contain said antiserum is an indication of high bactericidal activity.

2. The method of claim 1, wherein said color pH indicator is phenol red.

3. A method for detection of Lyme borreliosis, comprising

(a) contacting an antiserum against *B. burgdorferi* with a suitable color pH indicator for measurement of bacterial growth, and *B. burgdorferi* in a medium which is compatible for the growth of *B. burgdorferi* in the absence of said antiserum; and

(b) measuring the absorbance of the sample;

wherein high absorbance, when compared to a control sample which does not contain *B. burgdorferi* is an indication that *B. burgdorferi* is present in said suspected sample.

4. The method of claim 3, wherein said color pH indicator is phenol red.

5. A vaccine comprising OspA, OspB or fragments thereof; and a saponin adjuvant.

6. The vaccine of claim 5, further comprising a pharmaceutically acceptable carrier.

7. The vaccine of claim 5, further comprising a non-saponin adjuvant.

8. The vaccine of claim 5, wherein said saponin is QS-21.

9. The vaccine of claim 5, wherein said OspA and OspB are full length and lipidated.

10. The vaccine of claim 9, wherein said saponin is QS-21.

11. The vaccine of claim 9, wherein both the lipidated full-length OspA and the lipidated full-length OspB are present.

12. The vaccine of claim 11, wherein said saponin is QS-21.

13. A method of inducing immunity to bacteria causing Lyme disease in an animal, comprising administering to the animal a vaccine comprising OspA, OspB or fragments thereof; and a saponin adjuvant.

14. The method of claim 13, wherein said saponin is QS-21.

15. The method of claim 13, wherein said OspA and OspB are full length and lipidated.

16. The method of claim 15, wherein said saponin is QS-21.

17. The method of claim 15, wherein both lipidated full-length OspA and the lipidated full-length OspB are present in said vaccine.

18. The method of claim 17, wherein said saponin is QS-21.

19. The method of claim 13, wherein said bacteria are *B. burgdorferi*.

20. The method of claim 13, wherein said bacteria are *B. garinii*.

21. The method of claim 13, wherein said *B. burgdorferi* bacteria are strains CA-2-87 or B-31.

22. The method of claim 20, wherein said *B. garinii* is strain G-25.

23. The method of claim 13, wherein said bacteria are 24008 Fr.

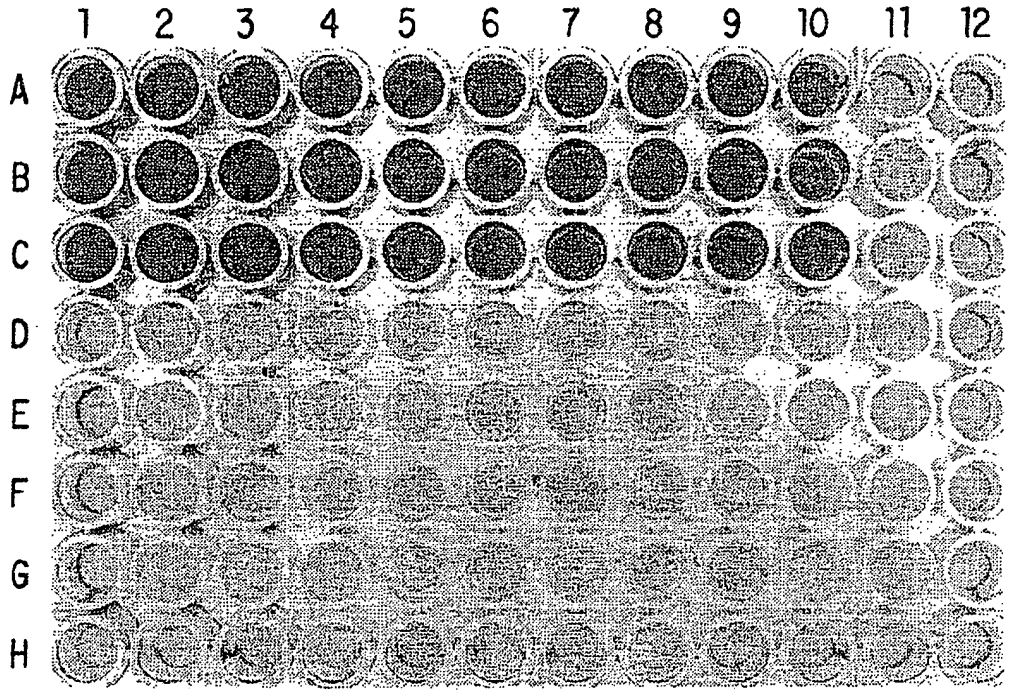

FIG. 1

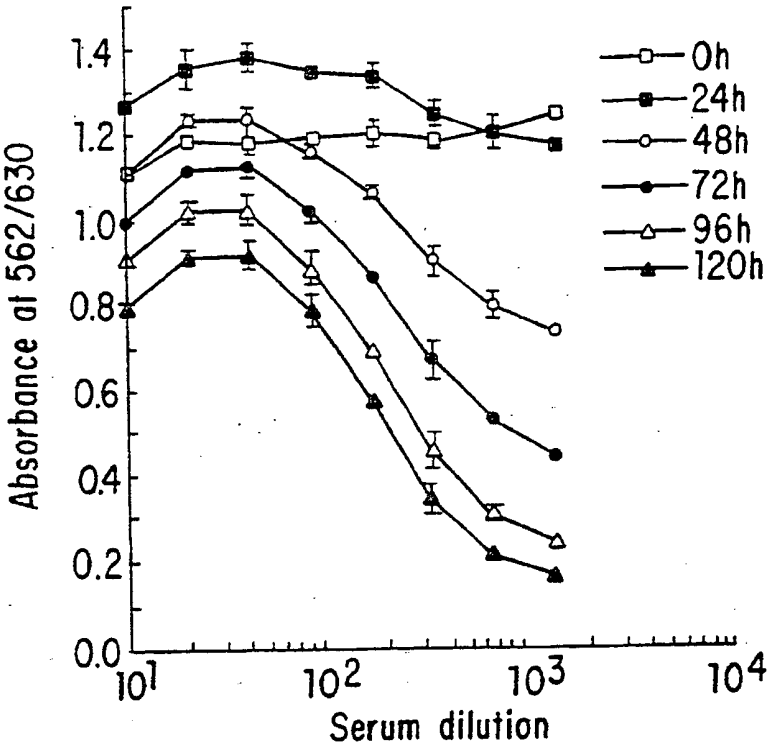
Absorbance at 562/630
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^1
10^2
10^3
10^4
Serum dilution
0h
24h
48h
72h
96h
120h

FIG. 2

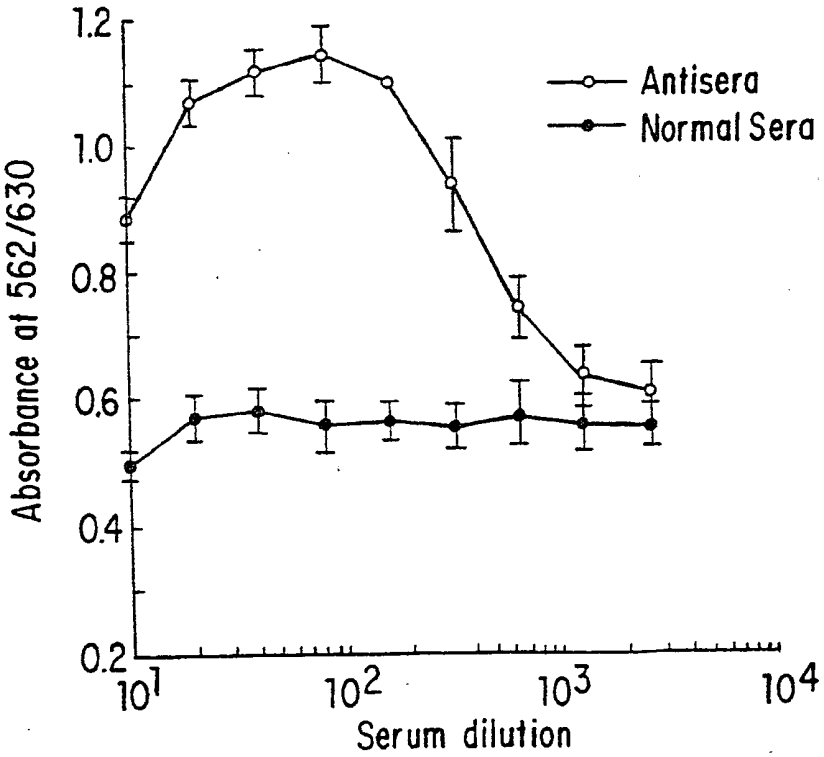
Absorbance at 562/630
1.2
1.0
0.8
0.6
0.4
0.2
10^1
10^2
10^3
10^4
Serum dilution
Antisera
Normal Sera

FIG. 3

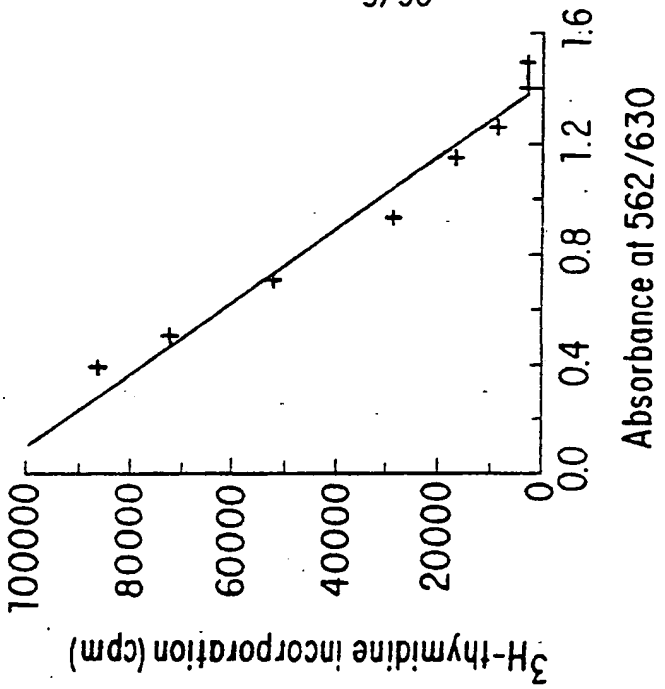
3H-thymidine incorporation (cpm)
100000
80000
60000
40000
20000
0
0.0
0.4
0.8
1.2
1.6
Absorbance at 562/630

FIG. 4B

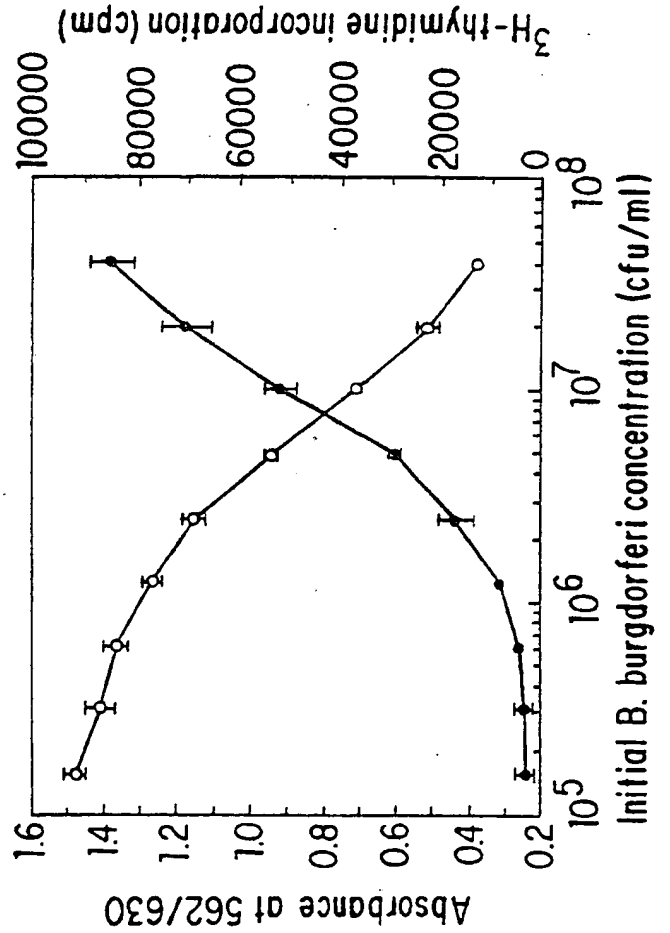
3H-thymidine incorporation (cpm)
100000
80000
60000
40000
20000
0
Absorbance at 562/630
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10^5
10^6
10^7
10^8
Initial B. burgdorferi concentration (cfu/ml)

FIG. 4A

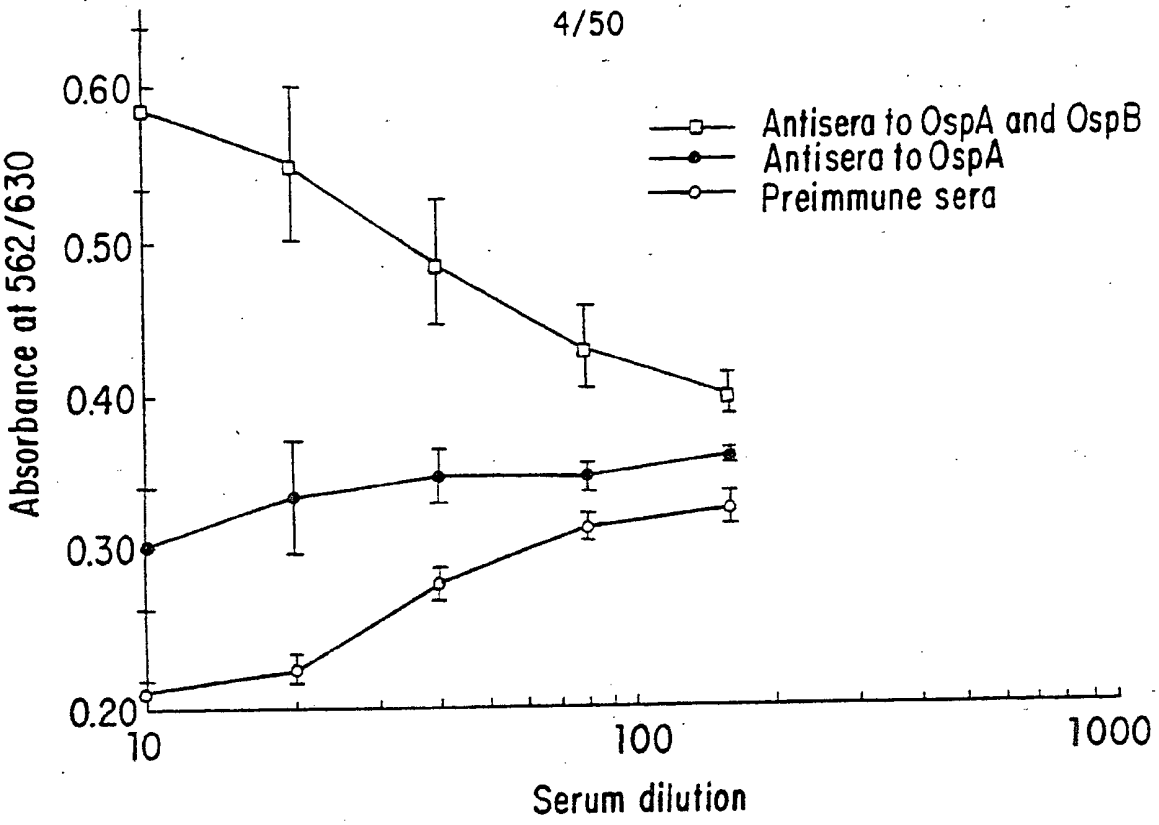
Antisera to OspA and OspB
Antisera to OspA
Preimmune sera
Absorbance at 562/630
0.60
0.50
0.40
0.30
0.20
10
100
1000
Serum dilution

FIG. 5

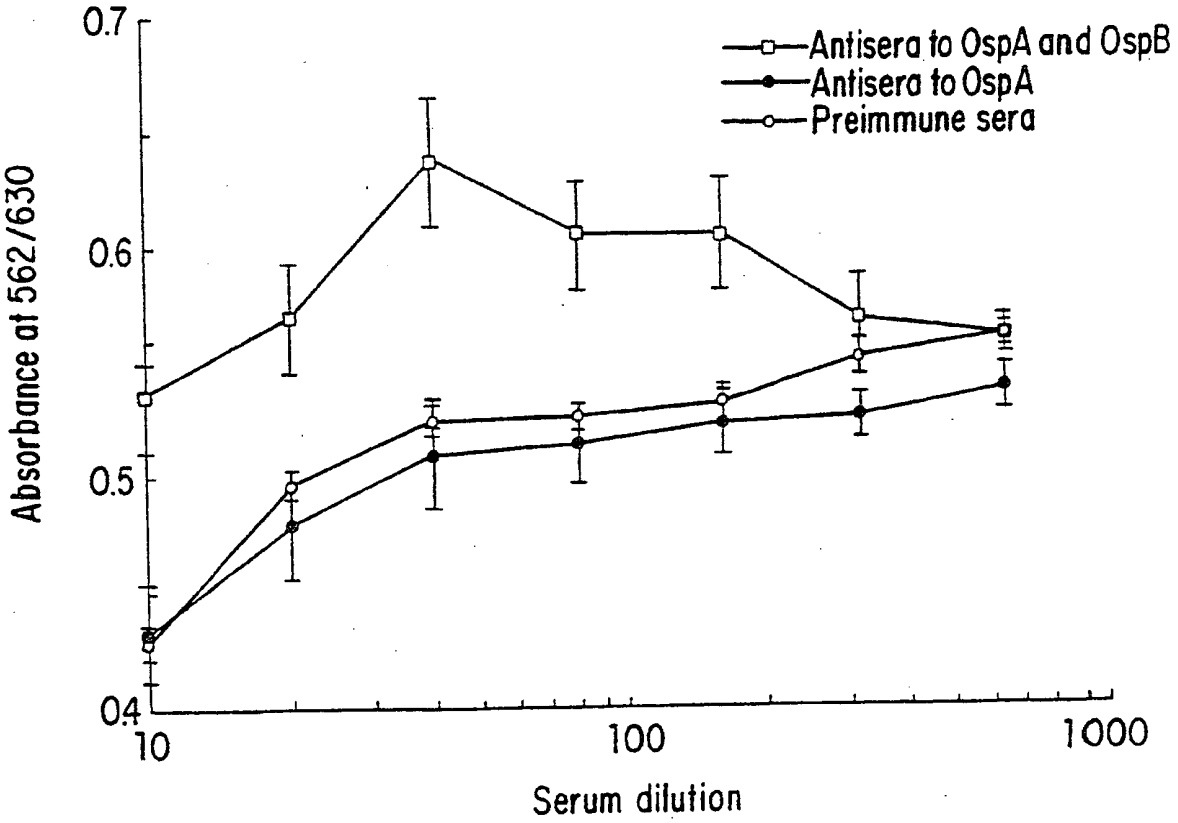
Antisera to OspA and OspB
Antisera to OspA
Preimmune sera
Absorbance at 562/630
0.7
0.6
0.5
0.4
10
100
1000
Serum dilution

FIG. 6

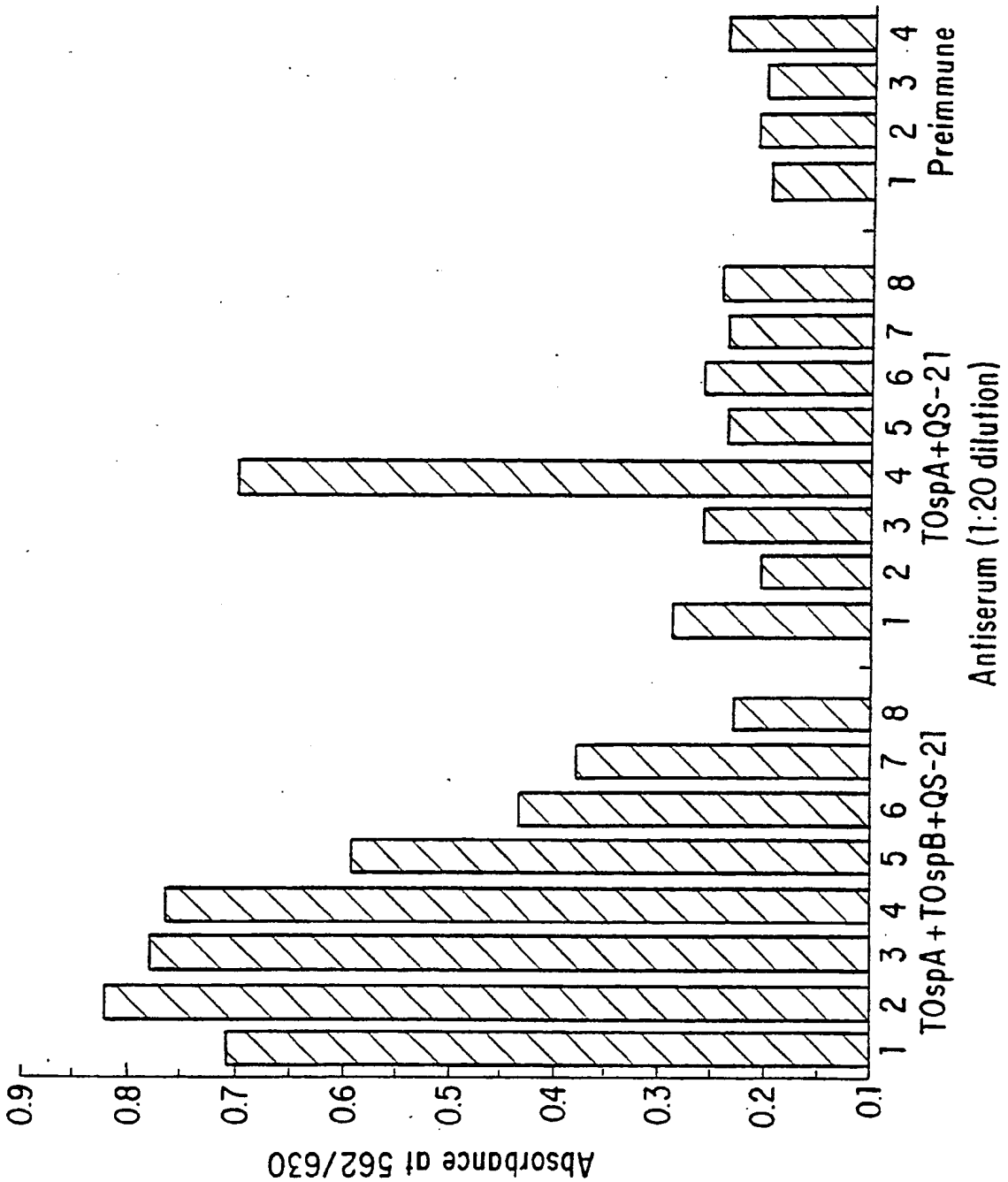
Absorbance at 562/630
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1 2 3 4 5 6 7 8
TOspA+TOspB+QS-21
1 2 3 4 5 6 7 8
TOspA+QS-21
1 2 3 4
Preimmune
Antiserum (1:20 dilution)

FIG. 7

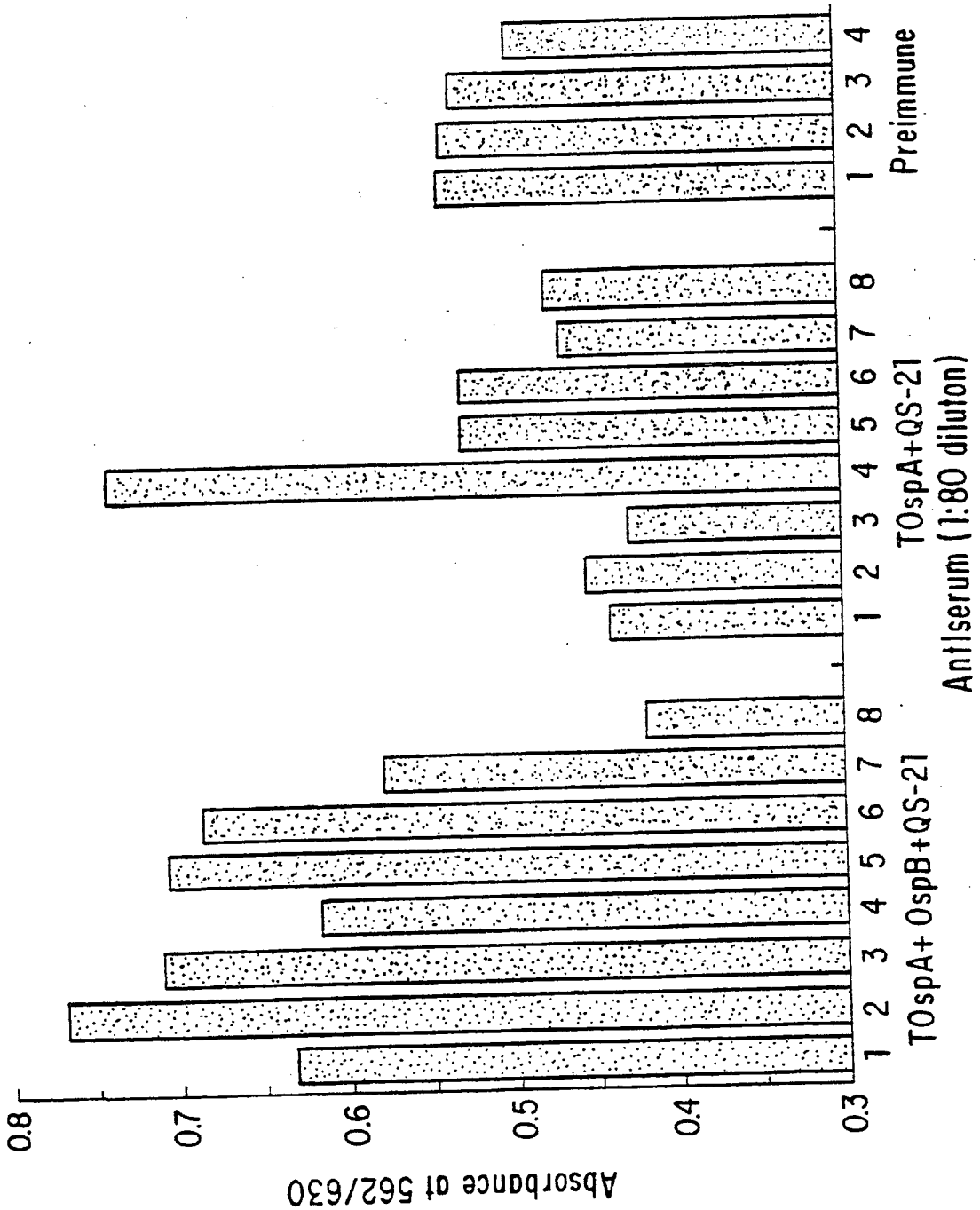
Absorbance at 562/630
0.8
0.7
0.6
0.5
0.4
0.3
1 2 3 4 5 6 7 8
TOspA+OspB+QS-21
1 2 3 4 5 6 7 8
TOspA+QS-21
Antiserum (1:80 dilution)
1 2 3 4
Preimmune

FIG. 8

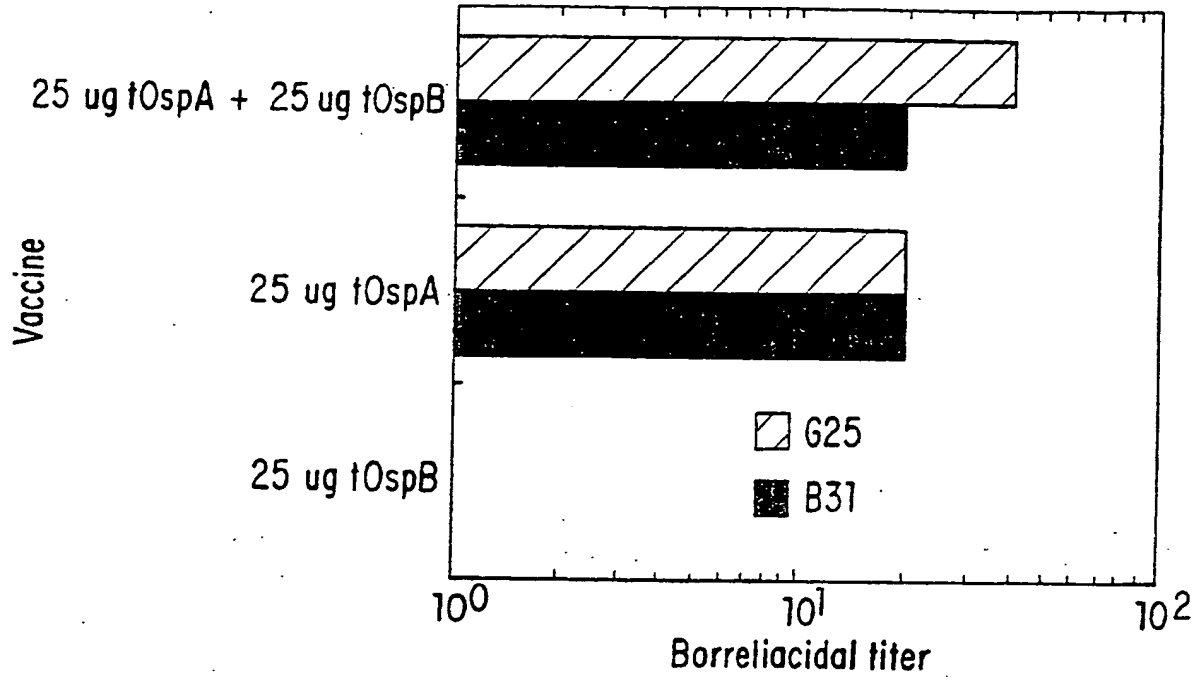
25 ug tOspA + 25 ug tOspB
25 ug tOspA
25 ug tOspB
Vaccine
G25
B31
10^0
10^1
10^2
Borreliacidal titer

FIG. 9

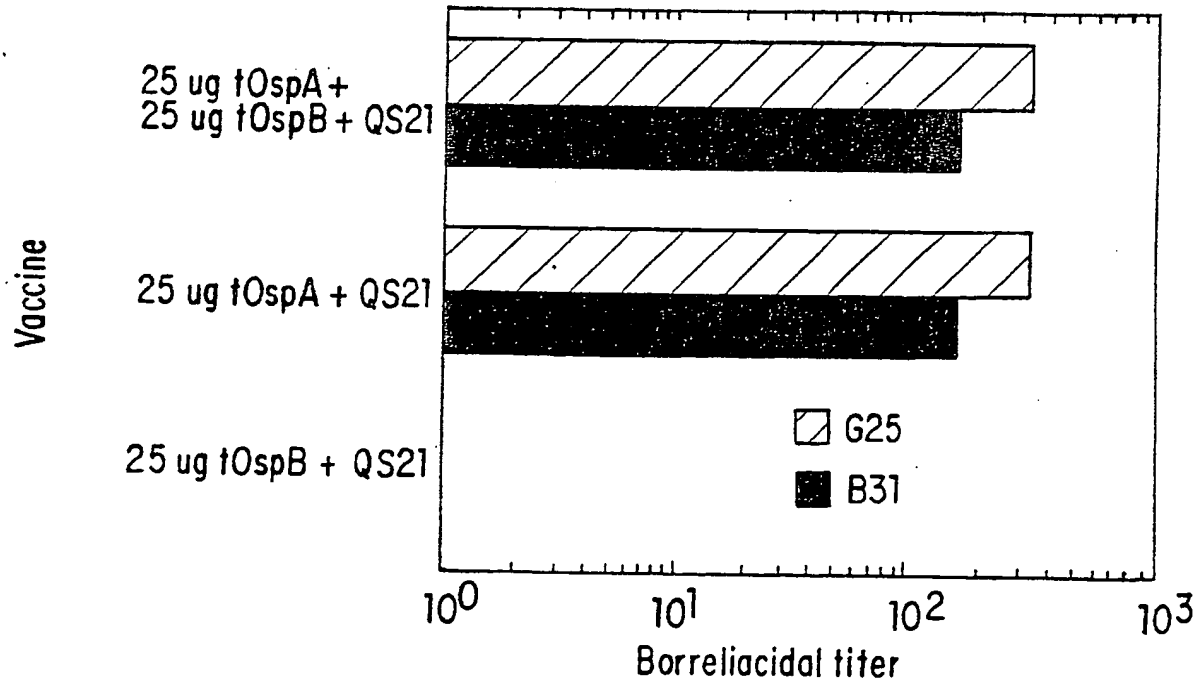
25 ug tOspA+
25 ug tOspB + QS21
25 ug tOspA + QS21
25 ug tOspB + QS21
Vaccine
G25
B31
10^0
10^1
10^2
10^3
Borreliacidal titer

FIG. 10

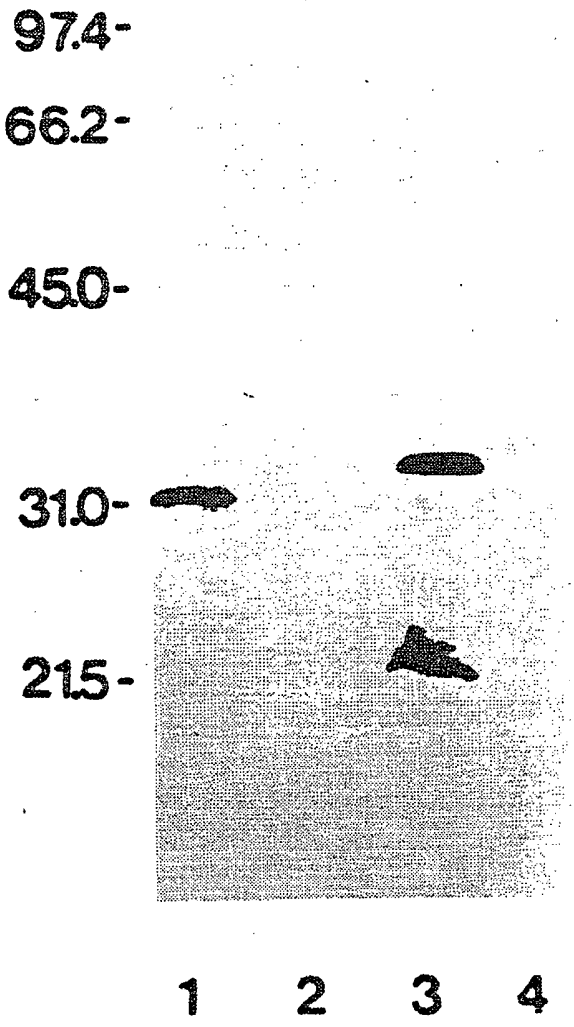

FIG. 11

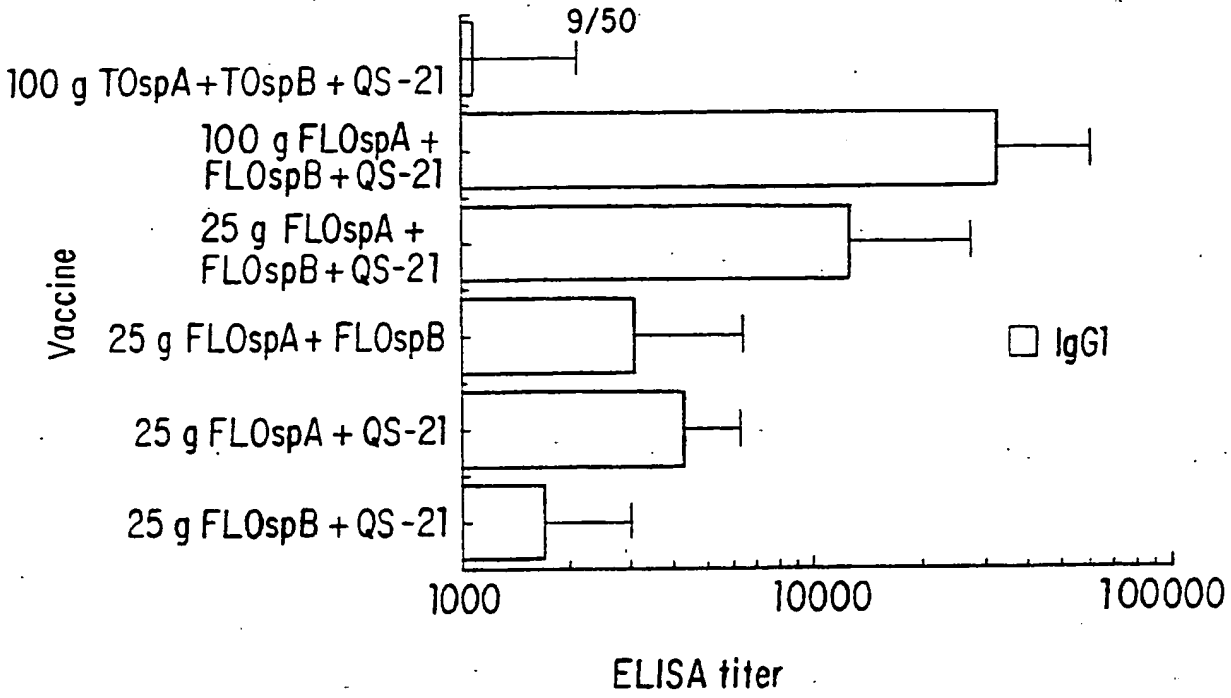

FIG. 12A

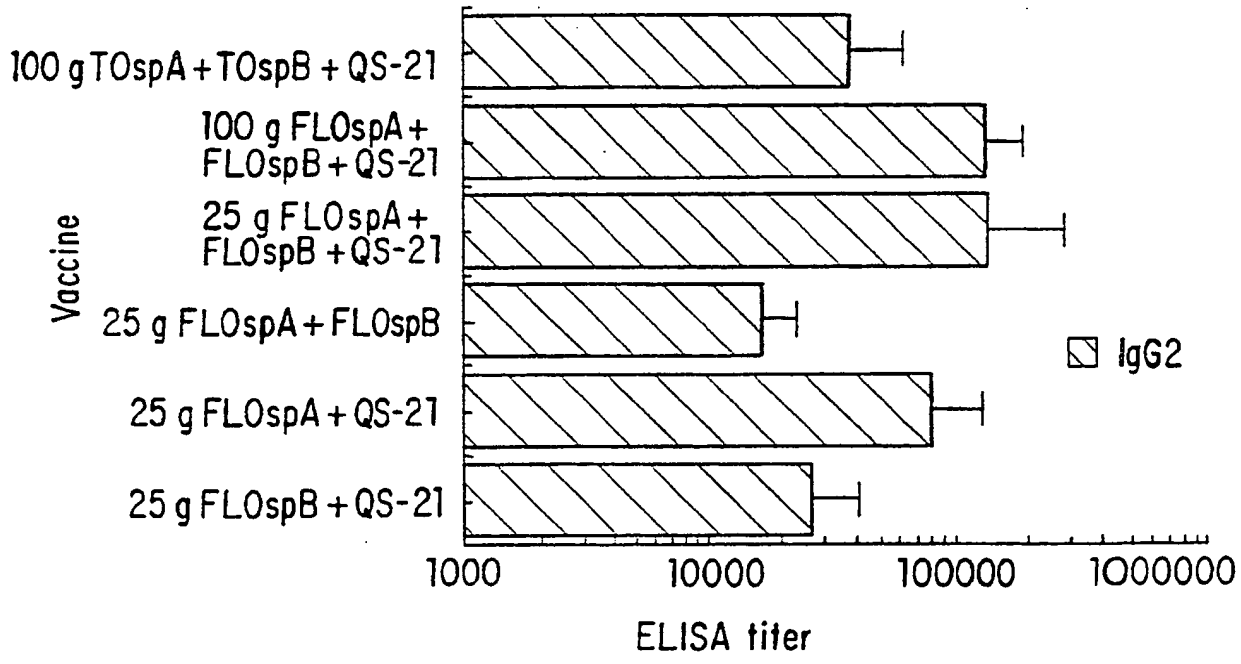

FIG. 12B

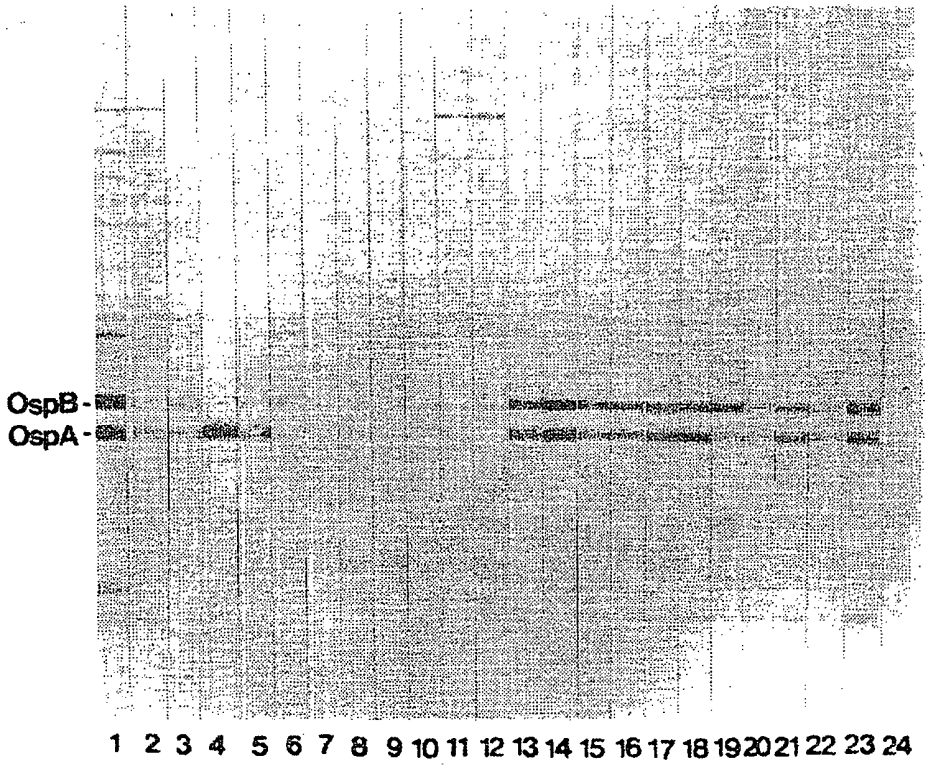

FIG. 13

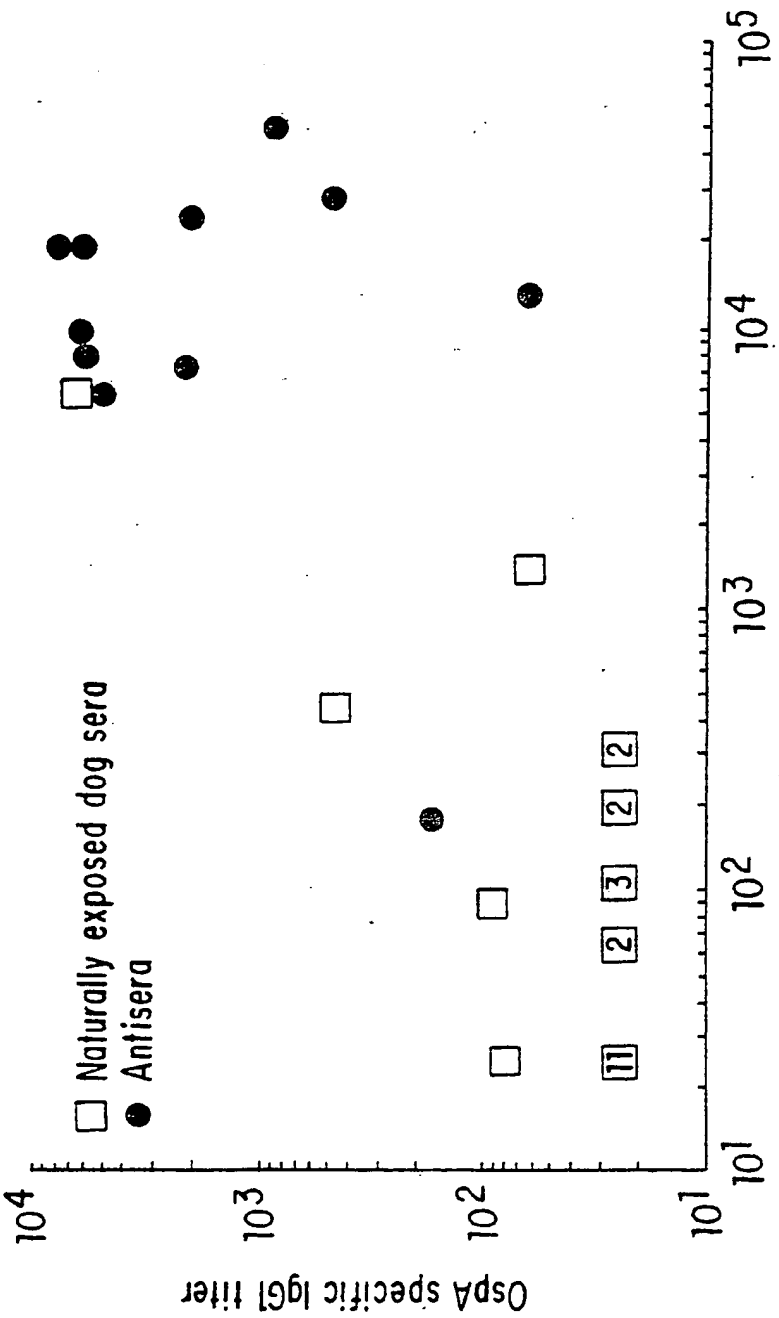
OspA specific IgG1 titer
OspA specific IgG2 titer
Naturally exposed dog sera
Antisera
10^1
10^2
10^3
10^4
10^5
11
2
3
2
2
2

FIG. 14A

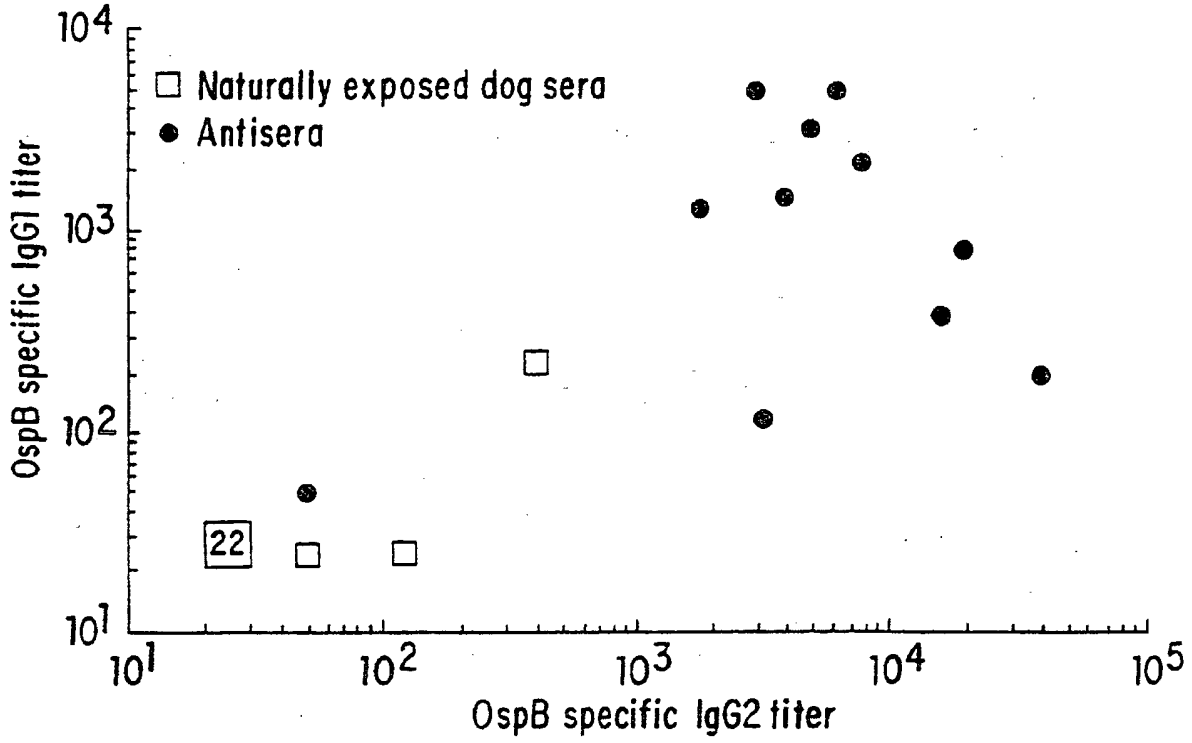
Naturally exposed dog sera
Antisera
OspB specific IgG1 titer
OspB specific IgG2 titer
22
10^1
10^2
10^3
10^4
10^5

FIG. 14B

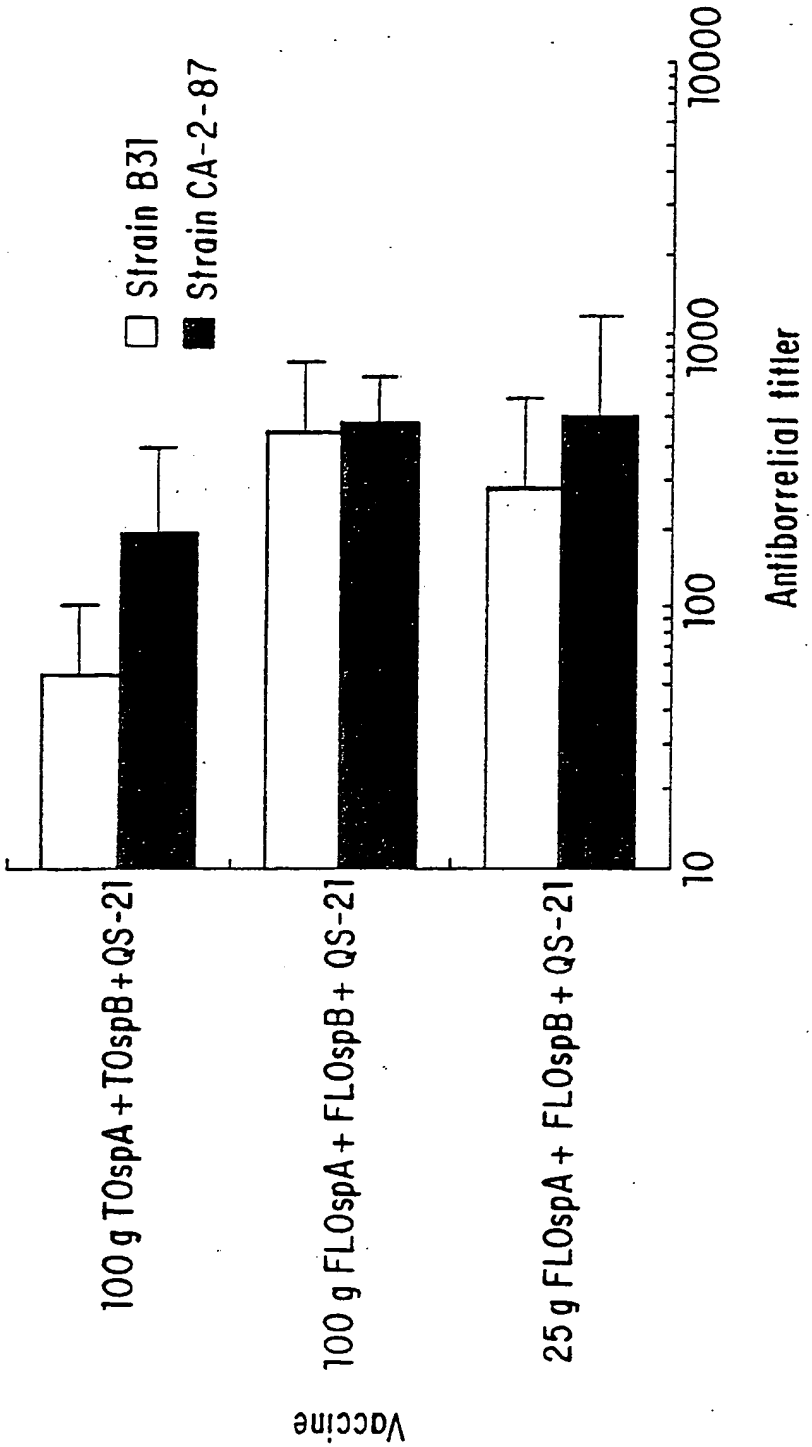
Strain B31
Strain CA-2-87
10000
1000
100
10
Antiborrelial titler
100 g TOspA + TOspB + QS-21
100 g FLOspA + FLOspB + QS-21
25 g FLOspA + FLOspB + QS-21
Vaccine

FIG. 15

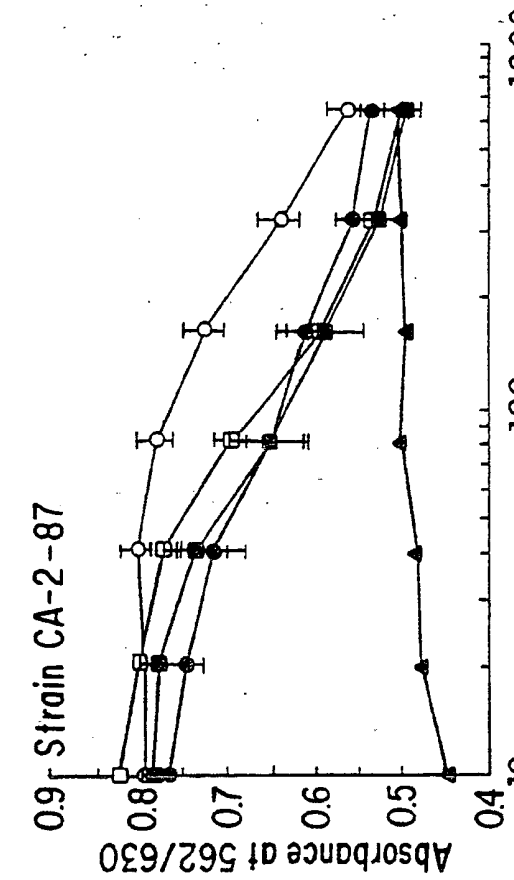

FIG. 16B

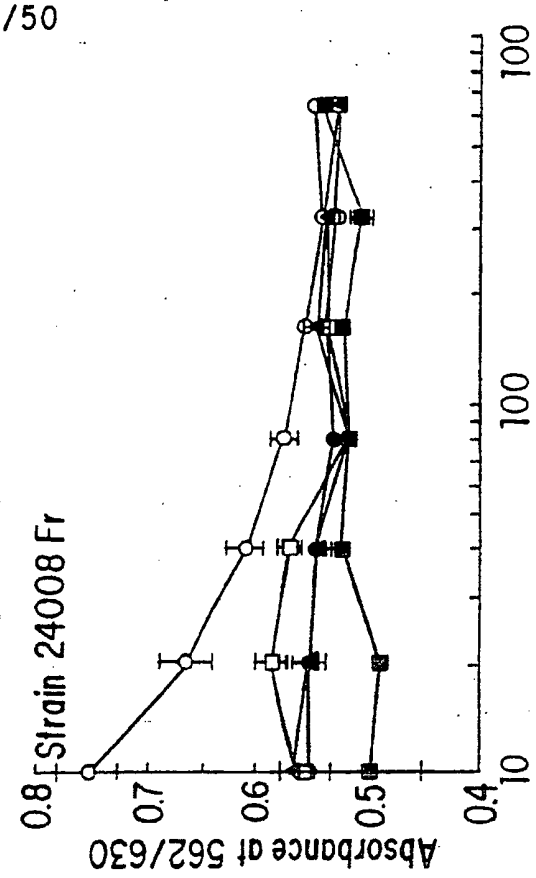

FIG. 16D

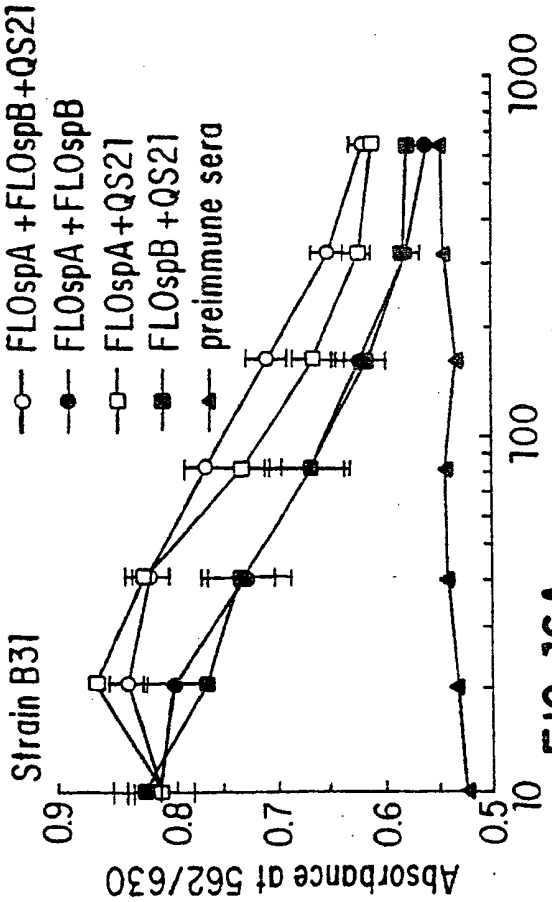

FIG. 16A

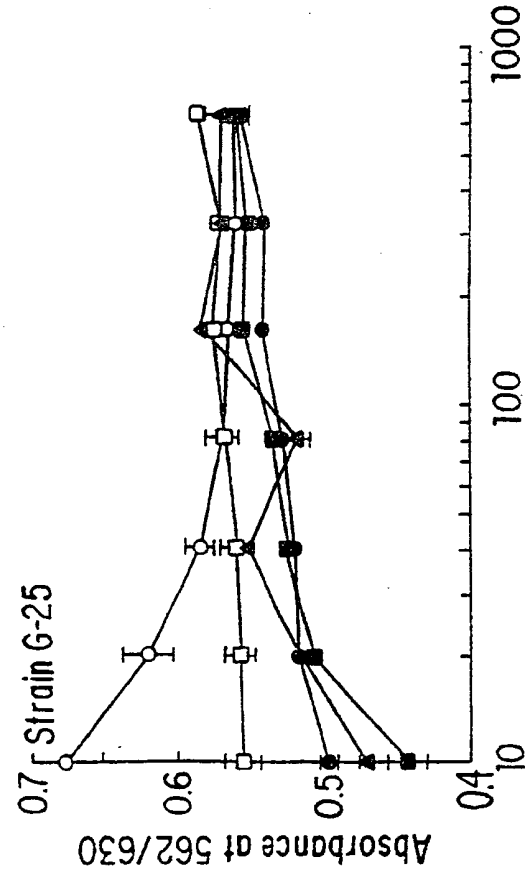

FIG. 16C

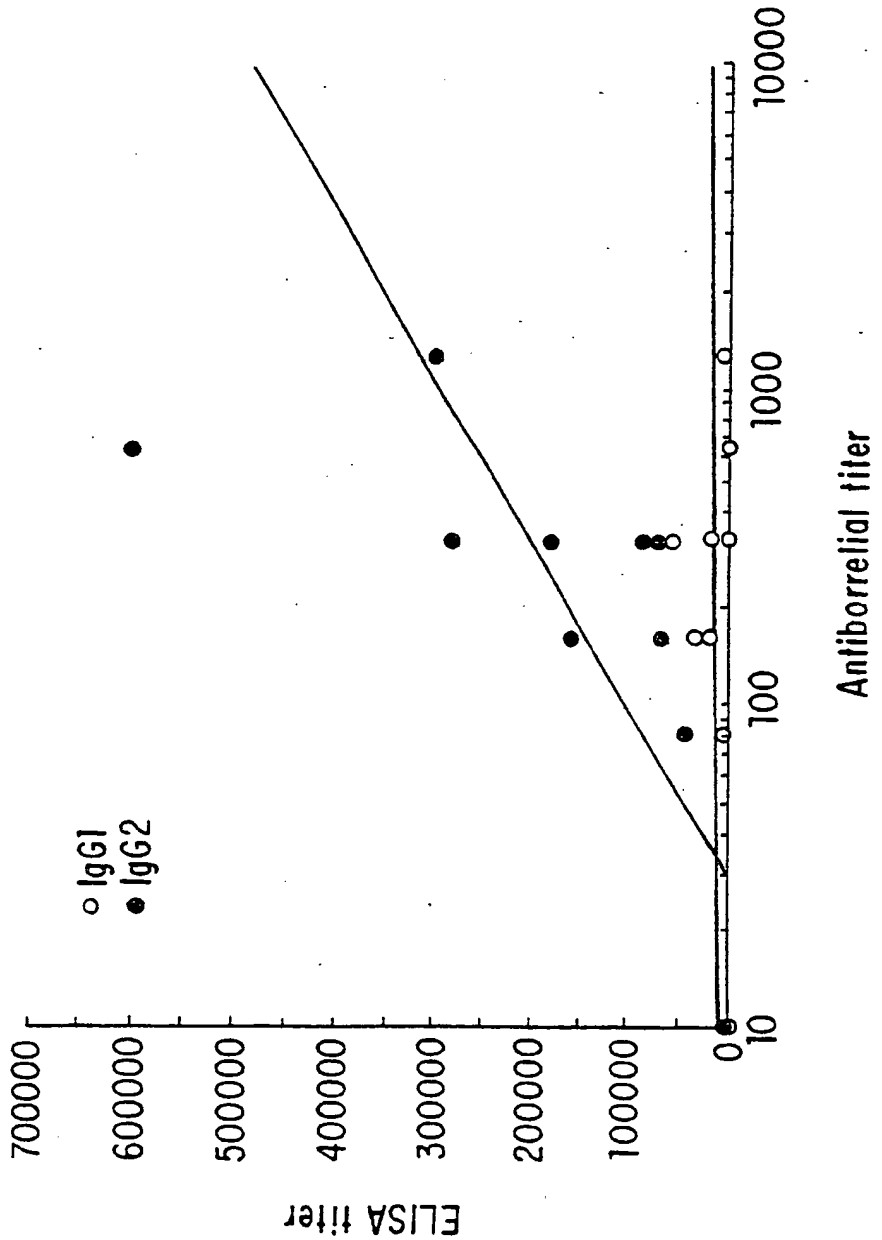
ELISA titer
700000
600000
500000
400000
300000
200000
100000
0
10
100
1000
10000
Antiborrelial titer
IgG1
IgG2

FIG. 17

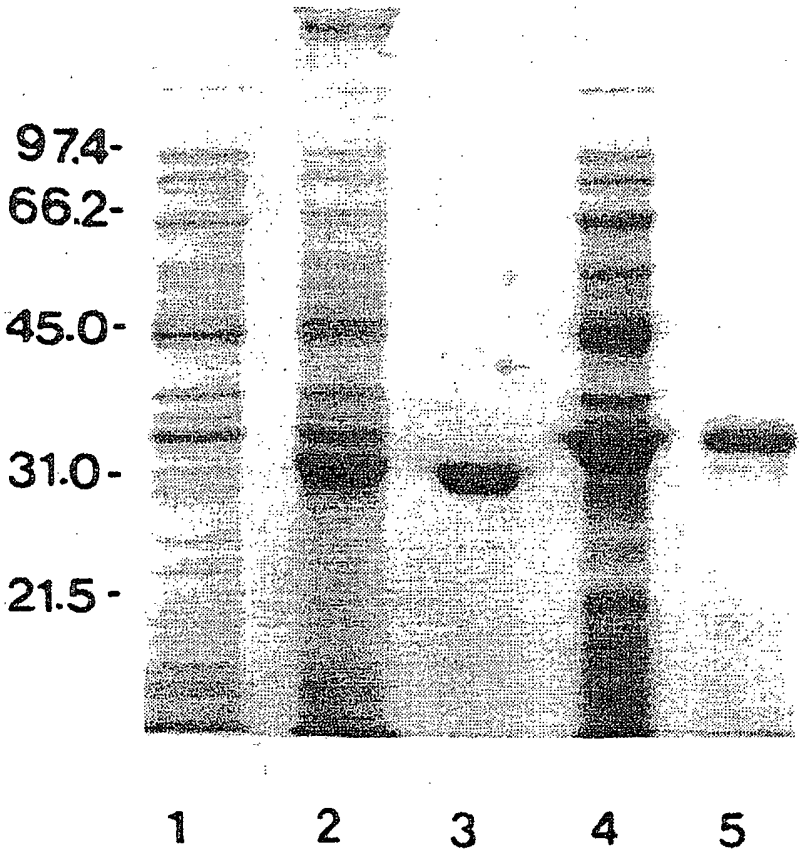

FIG. 18

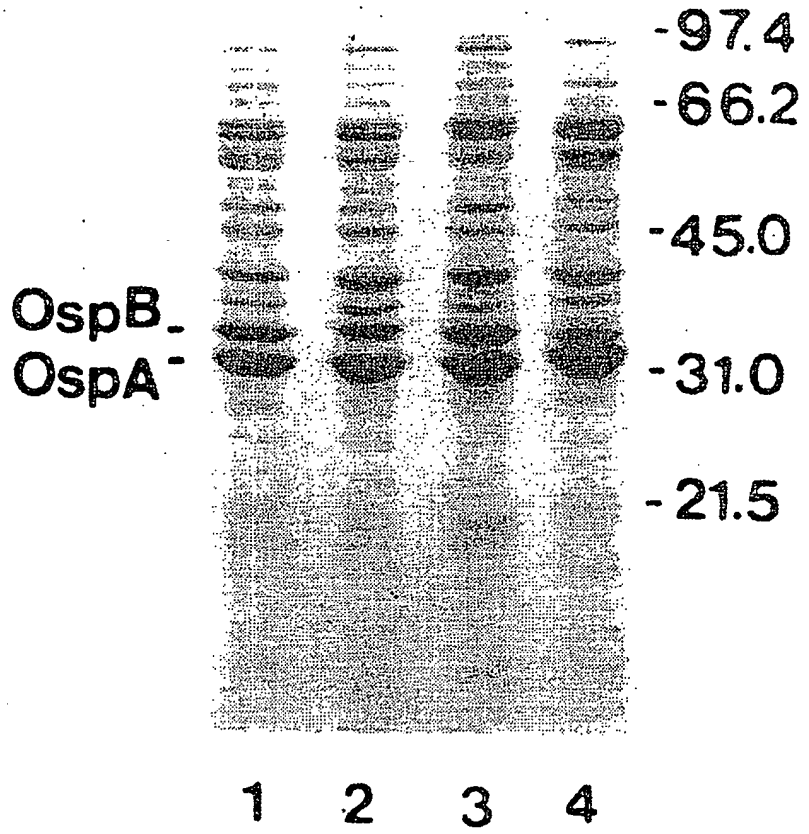

FIG. 19A

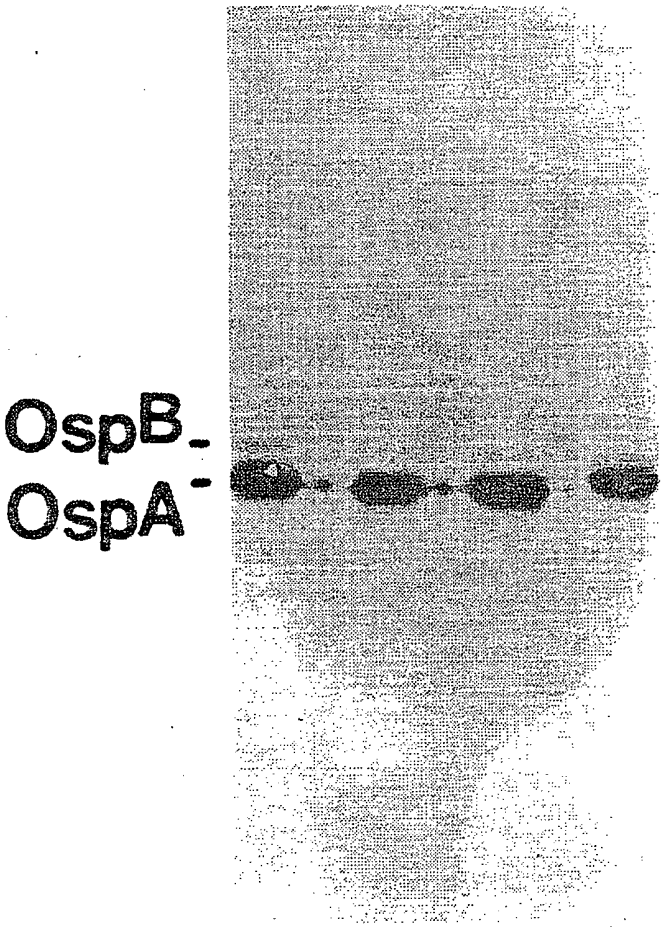

FIG. 19B

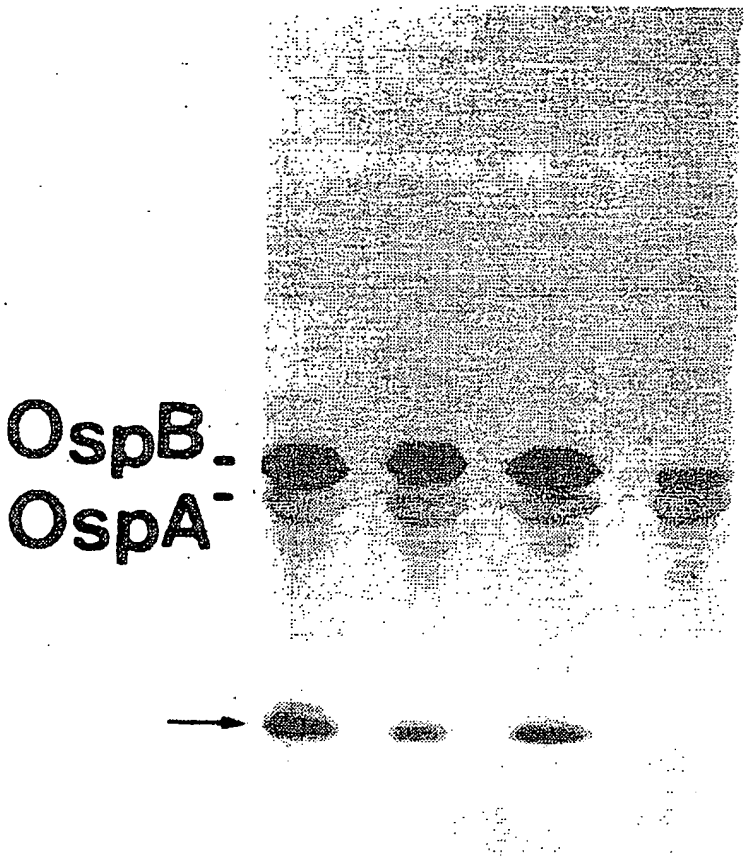

FIG. 19C

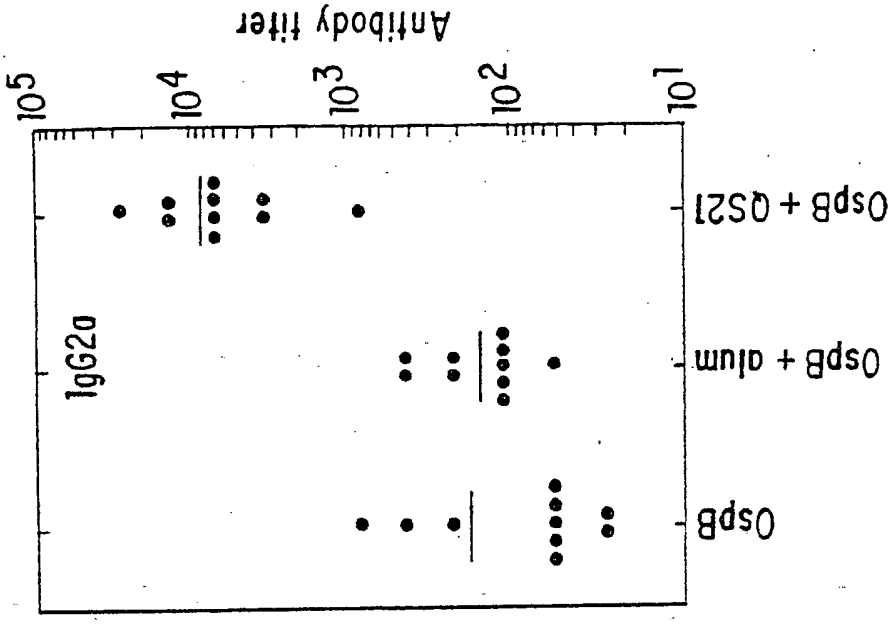

FIG. 20B

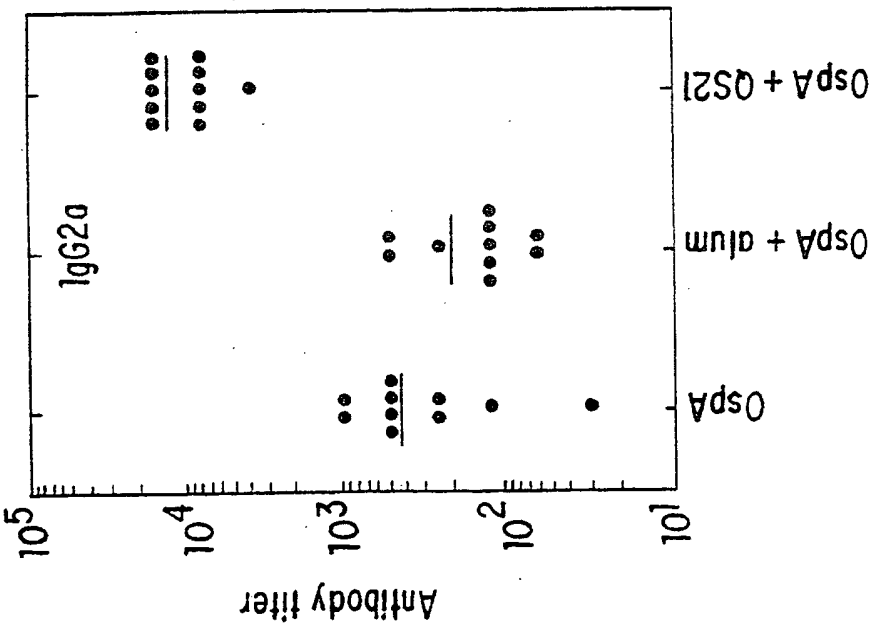

FIG. 20A

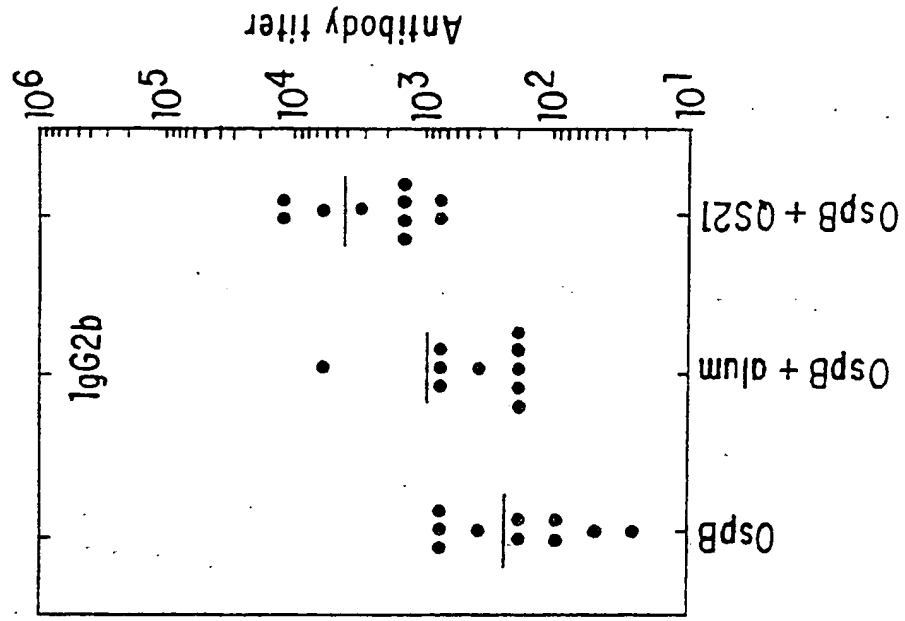

FIG. 20D

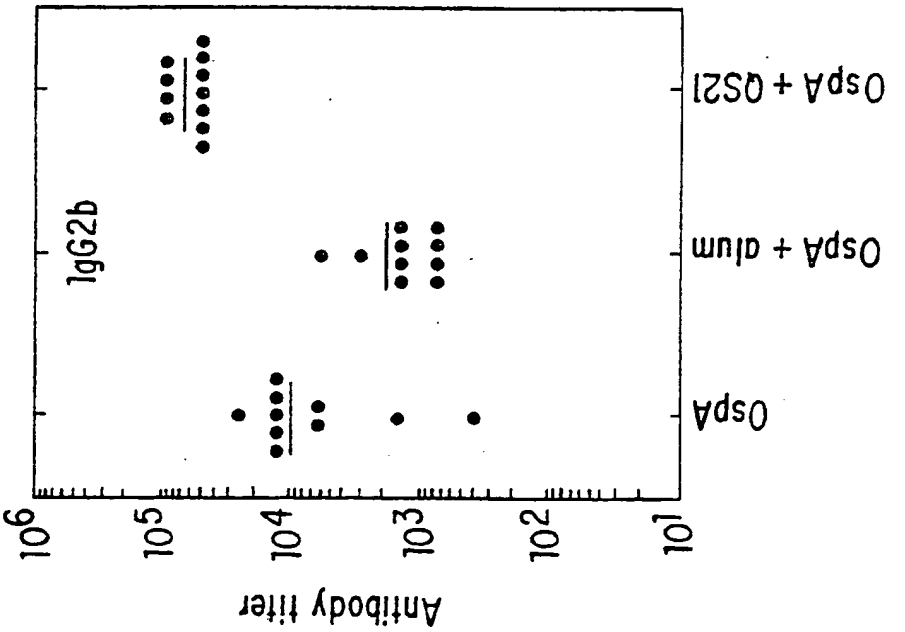

FIG. 20C

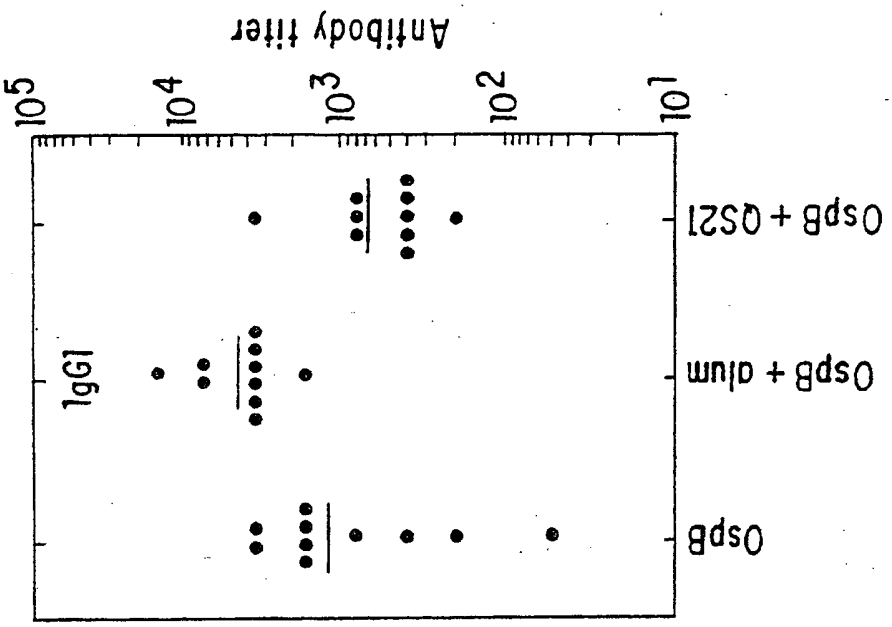

FIG. 20F

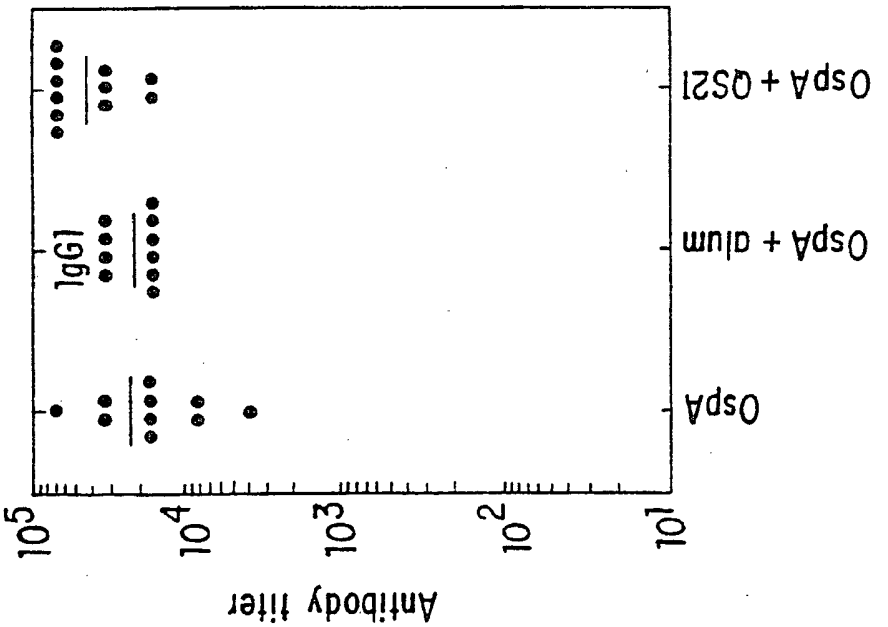

FIG. 20E

```
        10         20         30         40         50         60
         *          *          *          *          *          *
AAGCTTAATT AGAACCAAAC TTAATTAAAA CCAAACTTAA TTGAAGTTAT TATCATTTTA

        70         80         90        100        110        120
         *          *          *          *          *          *
TTTTTTTTCA ATTTTCTATT TGTTATTTGT TAATCTTATA ATATAATTAT ACTTGTATTA

       130        140        150        160        170
         *          *          *          *          *
AGTTATATTA ATATAAAAGG AGAATATATT ATG AAA AAA TAT TTA TTG GGA ATA
                                 Met Lys Lys Tyr Leu Leu Gly Ile>
                                 ___o___o__OspA ORF ___o___o___>

    180        190        200        210        220
     *          *          *          *          *
GGT CTA ATA TTA GCC TTA ATA GCA TGT AAG CAA AAT GTT AGC AGC CTT
Gly Leu Ile Leu Ala Leu Ile Ala Cys Lys Gln Asn Val Ser Ser Leu>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

       230        240        250        260        270
         *          *          *          *          *
GAC GAG AAA AAC AGC GTT TCA GTA GAT TTG CCT GGT GAA ATG AAA GTT
Asp Glu Lys Asn Ser Val Ser Val Asp Leu Pro Gly Glu Met Lys Val>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

           280        290        300        310
            *          *          *          *
CTT GTA AGC AAA GAA AAA AAC AAA GAC GGC AAG TAC GAT CTA ATT GCA
Leu Val Ser Lys Glu Lys Asn Lys Asp Gly Lys Tyr Asp Leu Ile Ala>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

320        330        340        350        360
 *          *          *          *          *
ACA GTA GAC AAG CTT GAG CTT AAA GGA ACT TCT GAT AAA AAC AAT GGA
Thr Val Asp Lys Leu Glu Leu Lys Gly Thr Ser Asp Lys Asn Asn Gly>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

  370        380        390        400        410
   *          *          *          *          *
TCT GGA GTA CTT GAA GGC GTA AAA GCT GAC AAA AGT AAA GTA AAA TTA
Ser Gly Val Leu Glu Gly Val Lys Ala Asp Lys Ser Lys Val Lys Leu>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>
```

FIG.21A

```
    420         430         440         450         460
     *           *           *           *           *
ACA ATT TCT GAC GAT CTA GGT CAA ACC ACA CTT GAA GTT TTC AAA GAA
Thr Ile Ser Asp Asp Leu Gly Gln Thr Thr Leu Glu Val Phe Lys Glu>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

       470         480         490         500         510
        *           *           *           *           *
GAT GGC AAA ACA CTA GTA TCA AAA AAA GTA ACT TCC AAA GAC AAG TCA
Asp Gly Lys Thr Leu Val Ser Lys Lys Val Thr Ser Lys Asp Lys Ser>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

            520         530         540         550
             *           *           *           *
TCA ACA GAA GAA AAA TTC AAT GAA AAA GGT GAA GTA TCT GAA AAA ATA
Ser Thr Glu Glu Lys Phe Asn Glu Lys Gly Glu Val Ser Glu Lys Ile>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___o>

560         570         580         590         600
 *           *           *           *           *
ATA ACA AGA GCA GAC GGA ACC AGA CTT GAA TAC ACA GGA ATT AAA AGC
Ile Thr Arg Ala Asp Gly Thr Arg Leu Glu Tyr Thr Gly Ile Lys Ser>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

  610         620         630         640         650
   *           *           *           *           *
GAT GGA TCT GGA AAA GCT AAA GAG GTT TTA AAA GGC TAT GTT CTT GAA
Asp Gly Ser Gly Lys Ala Lys Glu Val Leu Lys Gly Tyr Val Leu Glu>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

    660         670         680         690         700
     *           *           *           *           *
GGA ACT CTA ACT GCT GAA AAA ACA ACA TTG GTG GTT AAA GAA GGA ACT
Gly Thr Leu Thr Ala Glu Lys Thr Thr Leu Val Val Lys Glu Gly Thr>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

       710         720         730         740         750
        *           *           *           *           *
GTT ACT TTA AGC AAA AAT ATT TCA AAA TCT GGG GAA GTT TCA GTT GAA
Val Thr Leu Ser Lys Asn Ile Ser Lys Ser Gly Glu Val Ser Val Glu>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>
```

FIG.21B

```
          760         770         780         790
           *           *           *           *
CTT AAT GAC ACT GAC AGT AGT GCT GCT ACT AAA AAA ACT GCA GCT TGG
Leu Asn Asp Thr Asp Ser Ser Ala Ala Thr Lys Lys Thr Ala Ala Trp>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

800         810         820         830         840
 *           *           *           *           *
AAT TCA GGC ACT TCA ACT TTA ACA ATT ACT GTA AAC AGT AAA AAA ACT
Asn Ser Gly Thr Ser Thr Leu Thr Ile Thr Val Asn Ser Lys Lys Thr>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

  850         860         870         880         890
   *           *           *           *           *
AAA GAC CTT GTG TTT ACA AAA GAA AAC ACA ATT ACA GTA CAA CAA TAC
Lys Asp Leu Val Phe Thr Lys Glu Asn Thr Ile Thr Val Gln Gln Tyr>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

    900         910         920         930         940
     *           *           *           *           *
GAC TCA AAT GGC ACC AAA TTA GAG GGG TCA GCA GTT GAA ATT ACA AAA
Asp Ser Asn Gly Thr Lys Leu Glu Gly Ser Ala Val Glu Ile Thr Lys>
___o___o___o___o___o___o__OspA ORF ___o___o___o___o___o___o___>

      950         960         970         980         990
       *           *           *           *           *
CTT GAT GAA ATT AAA AAC GCT TTA AAA T AAGGAGAATT T ATG AGA TTA TTA
Leu Asp Glu Ile Lys Asn Ala Leu Lys>
___o___o____OspA ORF_o___o___o___>
                                                  Met Arg Leu Leu>
                                                  ___b___b___b___>

   1000        1010        1020        1030        1040
     *           *           *           *           *
ATA GGA TTT GCT TTA GCG TTA GCT TTA ATA GGA TGT GCA CAA AAA GGT
Ile Gly Phe Ala Leu Ala Leu Ala Leu Ile Gly Cys Ala Gln Lys Gly>
___b___b___b___b___b___b__OspB ORF___b___b___b___b___b___b___>

      1050        1060        1070        1080
       *           *           *           *
GCT GAG TCA ATT GGT TCT CAA AAA GAA AAT GAT CTA AAC CTT GAA GAC
Ala Glu Ser Ile Gly Ser Gln Lys Glu Asn Asp Leu Asn Leu Glu Asp>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>
```

FIG.21C

```
1090        1100        1110        1120        1130
 *           *           *           *           *
TCT AGT AAA AAA TCA CAT CAA AAC GCT AAA CAA GAC CTT CCT GCG GTG
Ser Ser Lys Lys Ser His Gln Asn Ala Lys Gln Asp Leu Pro Ala Val>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1140        1150        1160        1170        1180
 *           *           *           *           *
ACA GAA GAC TCA GTG TCT TTG TTT AAT GGT AAT AAA ATT TTT GTA AGC
Thr Glu Asp Ser Val Ser Leu Phe Asn Gly Asn Lys Ile Phe Val Ser>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1190        1200        1210        1220        1230
 *           *           *           *           *
AAA GAA AAA AAT AGC TCC GGC AAA TAT GAT TTA AGA GCA ACA ATT GAT
Lys Glu Lys Asn Ser Ser Gly Lys Tyr Asp Leu Arg Ala Thr Ile Asp>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1240        1250        1260        1270        1280
 *           *           *           *           *
CAG GTT GAA CTT AAA GGA ACT TCC GAT AAA AAC AAT GGT TCT GGA ACC
Gln Val Glu Leu Lys Gly Thr Ser Asp Lys Asn Asn Gly Ser Gly Thr>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1290        1300        1310        1320
 *           *           *           *
CTT GAA GGT TCA AAG CCT GAC AAG AGT AAA GTA AAA TTA ACA GTT TCT
Leu Glu Gly Ser Lys Pro Asp Lys Ser Lys Val Lys Leu Thr Val Ser>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1330        1340        1350        1360        1370
 *           *           *           *           *
GCT GAT TTA AAC ACA GTA ACC TTA GAA GCA TTT GAT GCC AGC AAC CAA
Ala Asp Leu Asn Thr Val Thr Leu Glu Ala Phe Asp Ala Ser Asn Gln
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1380        1390        1400        1410        1420
 *           *           *           *           *
AAA ATT TCA AGT AAA GTT ACT AAA AAA CAG GGG TCA ATA ACA GAG GAA
Lys Ile Ser Ser Lys Val Thr Lys Lys Gln Gly Ser Ile Thr Glu Glu>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>
```

FIG.21D

```
1430        1440        1450        1460        1470
 *           *           *           *           *
ACT CTC AAA GCT AAT AAA TTA GAC TCA AAG AAA TTA ACA AGA TCA AAC
Thr Leu Lys Ala Asn Lys Leu Asp Ser Lys Lys Leu Thr Arg Ser Asn>
__b__b__b__b__b__b__OspB ORF__b__b__b__b__b__b__b__>

    1480        1490        1500        1510        1520
     *           *           *           *           *
GGA ACT ACA CTT GAA TAC TCA CAA ATA ACA GAT GCT GAC AAT GCT ACA
Gly Thr Thr Leu Glu Tyr Ser Gln Ile Thr Asp Ala Asp Asn Ala Thr>
__b__b__b__b__b__b__OspB ORF__b__b__b__b__b__b__b__>

      1530        1540        1550        1560
       *           *           *           *
AAA GCA GTA GAA ACT CTA AAA AAT AGC ATT AAG CTT GAA GGA AGT CTT
Lys Ala Val Glu Thr Leu Lys Asn Ser Ile Lys Leu Glu Gly Ser Leu
__b__b__b__b__b__b__OspB ORF__b__b__b__b__b__b__b__>

1570        1580        1590        1600        1610
 *           *           *           *           *
GTA GTC GGA AAA ACA ACA GTG GAA ATT AAA GAA GGT ACT GTT ACT CTA
Val Val Gly Lys Thr Thr Val Glu Ile Lys Glu Gly Thr Val Thr Leu>
__b__b__b__b__b__b__OspB ORF__b__b__b__b__b__b__b__>

1620        1630        1640        1650        1660
 *           *           *           *           *
AAA AGA GAA ATT GAA AAA GAT GGA AAA GTA AAA GTC TTT TTG AAT GAC
Lys Arg Glu Ile Glu Lys Asp Gly Lys Val Lys Val Phe Leu Asn Asp>
__b__b__b__b__b__b__OspB ORF__b__b__b__b__b__b__b__>

  1670        1680        1690        1700        1710
   *           *           *           *           *
ACT GCA GGT TCT AAC AAA AAA ACA GGT AAA TGG GAA GAC AGT ACT AGC
Thr Ala Gly Ser Asn Lys Lys Thr Gly Lys Trp Glu Asp Ser Thr Ser>
__b__b__b__b__b__b__b__OspB ORF__b__b__b__b__b__b__>

    1720        1730        1740        1750        1760
     *           *           *           *           *
ACT TTA ACA ATT AGT GCT GAC AGC AAA AAA ACT AAA GAT TTG GTG TTC
Thr Leu Thr Ile Ser Ala Asp Ser Lys Lys Thr Lys Asp Leu Val Phe>
__b__b__b__b__b__b__OspB ORF__b__b__b__b__b__b__b__>
```

FIG.21E

```
      1770          1780          1790          1800
        *             *             *             *
TTA ACA GAT GGT ACA ATT ACA GTA CAA CAA TAC AAC ACA GCT GGA ACC
Leu Thr Asp Gly Thr Ile Thr Val Gln Gln Tyr Asn Thr Ala Gly Thr>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1810          1820          1830          1840          1850
 *             *             *             *             *
AGC CTA GAA GGA TCA GCA AGT GAA ATT AAA AAT CTT TCA GAG CTT AAA
Ser Leu Glu Gly Ser Ala Ser Glu Ile Lys Asn Leu Ser Glu Leu Lys>
___b___b___b___b___b___b___OspB ORF___b___b___b___b___b___b___b___>

1860          1870          1880          1890          1900          1910
  *             *             *             *             *             *
AAC GCT TTA AAA T AATATATAAG TAAACCCCCT ACAAGGCATC AGCTAGTGTA
Asn Ala Leu Lys>
___b___b___b___>

GGAAG
```

FIG.21F

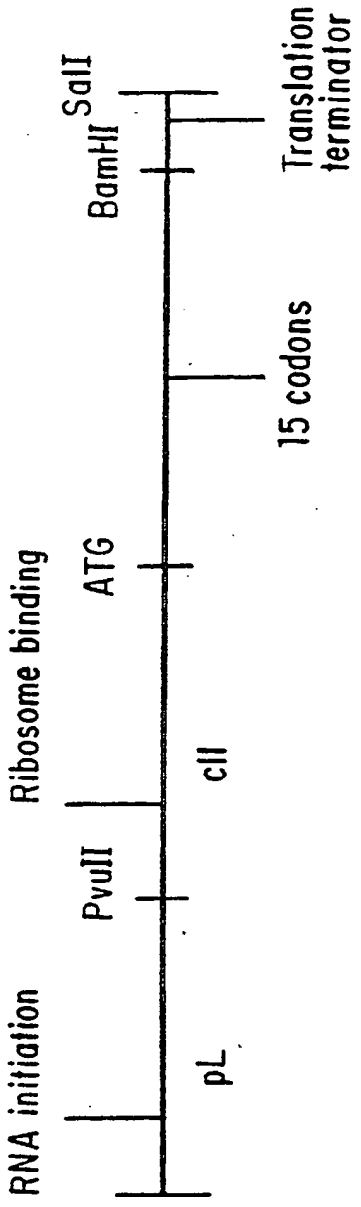
RNA initiation
pL
PvuII
cII
Ribosome binding
ATG
15 codons
BamHI
SalI
Translation terminator

FIG. 22

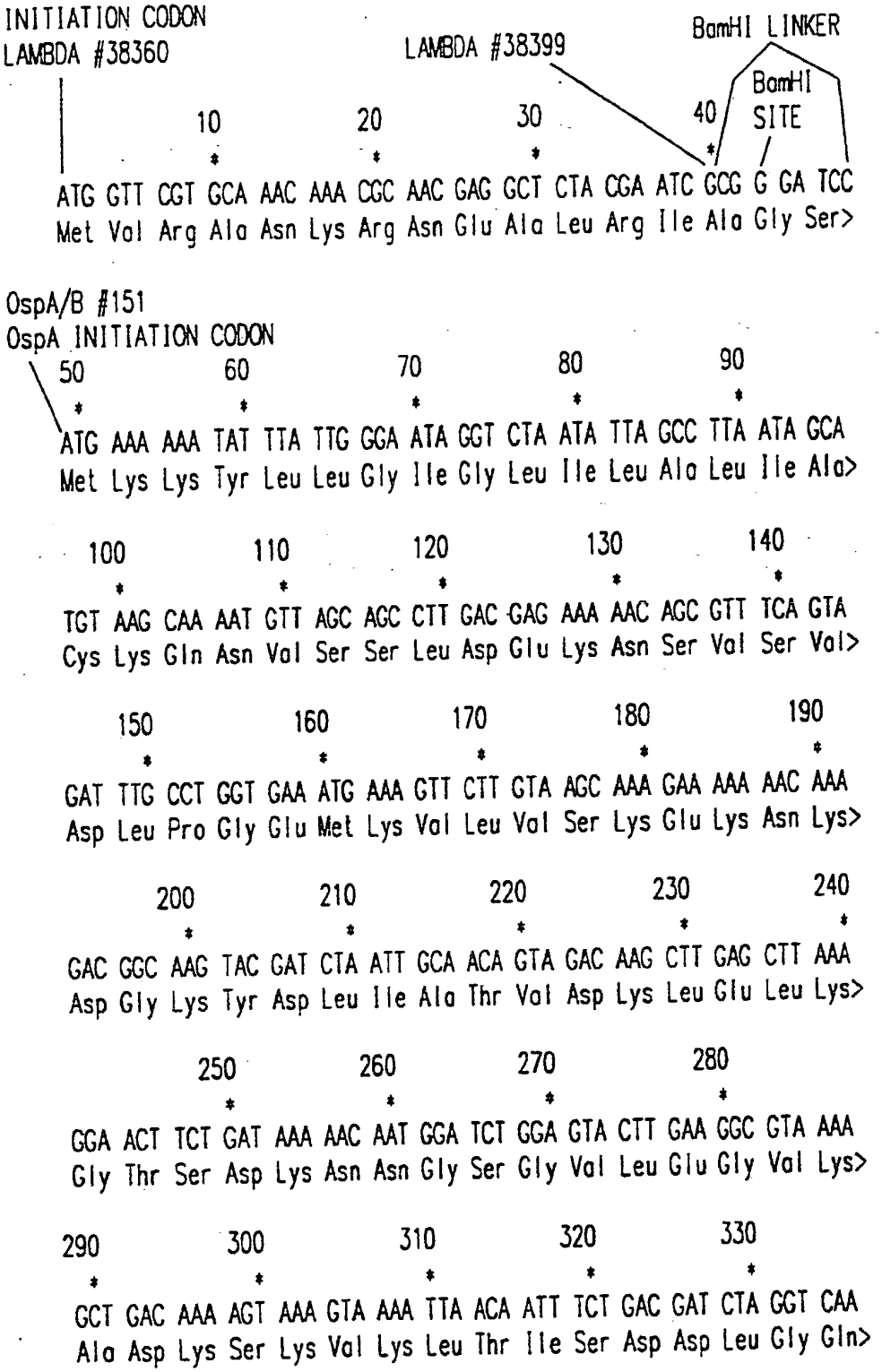
INITIATION CODON
LAMBDA #38360
LAMBDA #38399
BamHI LINKER
BamHI SITE
10 20 30 40
ATG GTT CGT GCA AAC AAA CGC AAC GAG GCT CTA CGA ATC GCG G GA TCC
Met Val Arg Ala Asn Lys Arg Asn Glu Ala Leu Arg Ile Ala Gly Ser>
OspA/B #151
OspA INITIATION CODON
50 60 70 80 90
ATG AAA AAA TAT TTA TTG GGA ATA GGT CTA ATA TTA GCC TTA ATA GCA
Met Lys Lys Tyr Leu Leu Gly Ile Gly Leu Ile Leu Ala Leu Ile Ala>
100 110 120 130 140
TGT AAG CAA AAT GTT AGC AGC CTT GAC GAG AAA AAC AGC GTT TCA GTA
Cys Lys Gln Asn Val Ser Ser Leu Asp Glu Lys Asn Ser Val Ser Val>
150 160 170 180 190
GAT TTG CCT GGT GAA ATG AAA GTT CTT GTA AGC AAA GAA AAA AAC AAA
Asp Leu Pro Gly Glu Met Lys Val Leu Val Ser Lys Glu Lys Asn Lys>
200 210 220 230 240
GAC GGC AAG TAC GAT CTA ATT GCA ACA GTA GAC AAG CTT GAG CTT AAA
Asp Gly Lys Tyr Asp Leu Ile Ala Thr Val Asp Lys Leu Glu Leu Lys>
250 260 270 280
GGA ACT TCT GAT AAA AAC AAT GGA TCT GGA GTA CTT GAA GGC GTA AAA
Gly Thr Ser Asp Lys Asn Asn Gly Ser Gly Val Leu Glu Gly Val Lys>
290 300 310 320 330
GCT GAC AAA AGT AAA GTA AAA TTA ACA ATT TCT GAC GAT CTA GGT CAA
Ala Asp Lys Ser Lys Val Lys Leu Thr Ile Ser Asp Asp Leu Gly Gln>

FIG.23A

```
340         350         360         370         380
 *           *           *           *           *
ACC ACA CTT GAA GTT TTC AAA GAA GAT GGC AAA ACA CTA GTA TCA AAA
Thr Thr Leu Glu Val Phe Lys Glu Asp Gly Lys Thr Leu Val Ser Lys>

   390         400         410         420         430
    *           *           *           *           *
AAA GTA ACT TCC AAA GAC AAG TCA TCA ACA GAA GAA AAA TTC AAT GAA
Lys Val Thr Ser Lys Asp Lys Ser Ser Thr Glu Glu Lys Phe Asn Glu>

      440         450         460         470         480
       *           *           *           *           *
AAA GGT GAA GTA TCT GAA AAA ATA ATA ACA AGA GCA GAC GGA ACC AGA
Lys Gly Glu Val Ser Glu Lys Ile Ile Thr Arg Ala Asp Gly Thr Arg>

         490         500         510         520
          *           *           *           *
CTT GAA TAC ACA GGA ATT AAA AGC GAT GGA TCT GGA AAA GCT AAA GAG
Leu Glu Tyr Thr Gly Ile Lys Ser Asp Gly Ser Gly Lys Ala Lys Glu>

530         540         550         560         570
 *           *           *           *           *
GTT TTA AAA GGC TAT GTT CTT GAA GGA ACT CTA ACT GCT GAA AAA ACA
Val Leu Lys Gly Tyr Val Leu Glu Gly Thr Leu Thr Ala Glu Lys Thr>

  580         590         600         610         620
   *           *           *           *           *
ACA TTG GTG GTT AAA GAA GGA ACT GTT ACT TTA AGC AAA AAT ATT TCA
Thr Leu Val Val Lys Glu Gly Thr Val Thr Leu Ser Lys Asn Ile Ser>

   630         640         650         660         670
    *           *           *           *           *
AAA TCT GGG GAA GTT TCA GTT GAA CTT AAT GAC ACT GAC AGT AGT GCT
Lys Ser Gly Glu Val Ser Val Glu Leu Asn Asp Thr Asp Ser Ser Ala>

      680         690         700         710         720
       *           *           *           *           *
GCT ACT AAA AAA ACT GCA GCT TGG AAT TCA GGC ACT TCA ACT TTA ACA
Ala Thr Lys Lys Thr Ala Ala Trp Asn Ser Gly Thr Ser Thr Leu Thr>
```

FIG.23B

```
        730         740         750         760
         *           *           *           *
ATT ACT GTA AAC AGT AAA AAA ACT AAA GAC CTT GTG TTT ACA AAA GAA
Ile Thr Val Asn Ser Lys Lys Thr Lys Asp Leu Val Phe Thr Lys Glu>

770         780         790         800         810
 *           *           *           *           *
AAC ACA ATT ACA GTA CAA CAA TAC GAC TCA AAT GGC ACC AAA TTA GAG
Asn Thr Ile Thr Val Gln Gln Tyr Asp Ser Asn Gly Thr Lys Leu Glu>

  820         830         840         850         860
   *           *           *           *           *
GGG TCA GCA GTT GAA ATT ACA AAA CTT GAT GAA ATT AAA AAC GCT TTA
Gly Ser Ala Val Glu Ile Thr Lys Leu Asp Glu Ile Lys Asn Ala Leu>

    870         880         890         900         910         920
     *           *           *           *           *           *
AAA TAA|GGAGAATTTA TGAGATTATT AATAGGATTT GCTTTAGCGT TAGCTTTAAT
Lys>
```

STOP CODON
FOR Osp A

POLY-TRANSLATION TERMINATION OLIGOMER

```
      930         940         950         960         970         980
       *           *           *           *           *           *
AGGATGTGCA CAAAAAGGTG CTGGG GATCC TAGGTAAGTA G G TCGACCGA TGCCCTTGAG
                          |                           |
                    OspA/B #1045                pBR322  #652

      990        1000        1010        1020        1030        1040
       *           *           *           *           *           *
AGCCTTCAAC CCAGTCAGCT CCTTCCGGTG GGCGCGGGGC ATGACTATCG TCGCCGCACT

     1050        1060        1070        1080        1090        1100
       *           *           *           *           *           *
TATGACTGTC TTCTTTATCA TGCAACTCGT AGGACAGGTG CCGGCAGCGC TCTGGGTCAT
```

FIG.23C

```
      1110        1120        1130        1140        1150        1160
       *           *           *           *           *           *
TTTCGGCGAG GACCGCTTTC GCTGGAGCGC GACGATGATC GGCCTGTCGC TTGCGGTATT

      1170        1180        1190        1200        1210        1220
       *           *           *           *           *           *
CGGAATCTTG CACGCCCTCG CTCAAGCCTT CGTCACTGGT CCCGCCACCA AACGTTTCGG

      1230        1240        1250        1260        1270        1280
       *           *           *           *           *           *
CGAGAAGCAG GCCATTATCG CCGGCATGGC GGCCGACGCG CTGGGCTACG TCTTGCTGGC
      1290        1300        1310        1320        1330        1340
       *           *           *           *           *           *
GTTCGCGACG CGAGGCTGGA TGGCCTTCCC CATTATGATT CTTCTCGCTT CCGGCGGCAT

      1350        1360        1370        1380        1390        1400
       *           *           *           *           *           *
CGGGATGCCC GCGTTGCAGG CCATGCTGTC CAGGCAGGTA GATGACGACC ATCAGGGACA

      1410        1420        1430        1440        1450        1460
       *           *           *           *           *           *
GCTTCAAGGA TCGCTCGCGG CTCTTACCAG CCTAACTTCG ATCACTGGAC CGCTGATCGT

      1470        1480        1490        1500        1510        1520
       *           *           *           *           *           *
CACGGCGATT TATGCCGCCT CGGCGAGCAC ATGGAACGGG TTGGCATGGA TTGTAGGCGC

      1530        1540        1550        1560        1570        1580
       *           *           *           *           *           *
CGCCCTATAC CTTGTCTGCC TCCCCGCGTT GCGTCGCGGT GCATGGAGCC GGGCCACCTC

      1590        1600        1610        1620        1630        1640
       *           *           *           *           *           *
GACCTGAATG GAAGCCGGCG GCACCTCGCT AACGGATTCA CCACTCCAAG AATTGGAGCC

      1650        1660        1670        1680        1690        1700
       *           *           *           *           *           *
AATCAATTCT TGCGGAGAAC TGTGAATGCG CAAACCAACC CTTGGCAGAA CATATCCATC
```

FIG.23D

pBR322#1446 pBR322#2069

```
      1710        1720        1730        1740        1750        1760
       *           *           *           *           *           *
GCGTCCGCCA TCTCCAGCAG CCGCACGCGG CGCATCTCGG GCAGCGTTGG GTCCTGG CTG

      1770        1780        1790        1800        1810        1820
       *           *           *           *           *           *
CCTCGCGCGT TTCGGTGATG ACGGTGAAAA CCTCTGACAC ATGCAGCTCC CGGAGACGGT

      1830        1840        1850        1860        1870        1880
       *           *           *           *           *           *
CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAGCC CGTCAGGGCG CGTCAGCGGG

      1890        1900        1910        1920        1930        1940
       *           *           *           *           *           *
TGTTGGCGGG TGTCGGGGCG CAGCCATGAC CCAGTCACGT AGCGATAGCG GAGTGTATAC

      1950        1960        1970        1980        1990        2000
       *           *           *           *           *           *
TGGCTTAACT ATGCGGCATC AGAGCAGATT GTACTGAGAG TGCACCATAT GCGGTGTGAA

      2010        2020        2030        3040        2050        2060
       *           *           *           *           *           *
ATACCGCACA GATGCGTAAG GAGAAAATAC CGCATCAGGC GCTCTTCCGC TTCCTCGCTC

      2070        2080        2090        2100        2110        2120
       *           *           *           *           *           *
ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG

      2130        2140        2150        2160        2170        2180
       *           *           *           *           *           *
GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC

      2180        2200        2210        2220        2230        2240
       *           *           *           *           *           *
CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC

      2250        2260        2270        2280        2290        2300
       *           *           *           *           *           *
CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA
```

FIG.23E

2310 2320 2330 2340 2350 2360
CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC

2370 2380 2390 2400 2410 2420
CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAA

2430 2440 2450 2460 2470 2480
TGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG

2490 2500 2510 2520 2530 2540
CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC

2550 2560 2570 2580 2590 2600
AACCCGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA

2610 2620 2630 2640 2650 2660
GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT

2670 2680 2690 2700 2710 2720
AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT

2730 2740 2750 2760 2770 2780
GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG

2790 2800 2810 2820 2830 2840
CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG

2850 2860 2870 2880 2890 2900
TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA

FIG.23F

```
      2910        2920        2930        2940        2950        2960
       *           *           *           *           *           *
AGGATCTTCA CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA

      2970        2980        2990        3000        3010
       *           *           *           *           *
TATGAGTAAA CTTGGTCTGA CAGTTA CCA ATG CTT AAT CAG TGA GGC ACC TAT

      3020        3030        3040        3050        3060
       *           *           *           *           *
CTC AGC GAT CTG TCT ATT TCG TTC ATC CAT AGT TGC CTG ACT CCC CGT

      3070        3080        3090        3100
       *           *           *           *
CGT GTA GAT AAC TAC GAT ACG GGA GGG CTT ACC ATC TGG CCC CAG TGC

3110        3120        3130        3140        3150
 *           *           *           *           *
TGC AAT GAT ACC GCG AGA CCC ACG CTC ACC GGC TCC AGA TTT ATC AGC

3160        3170        3180        3190        3200
 *           *           *           *           *
AAT AAA CCA GCC AGC CGG AAG GGC CGA GCG CAG AAG TGG TCC TGC AAC

      3210        3220        3230        3240        3250
       *           *           *           *           *
TTT ATC CGC CTC CAT CCA GTC TAT TAA TTG TTG CCG GGA AGC TAG AGT

      3260        3270        3280        3290        3300
       *           *           *           *           *
AAG TAG TTC GCC AGT TAA TAG TTT GCG CAA CGT TGT TGC CAT TGC TGC

      3310        3320        3330        3340
       *           *           *           *
AGG CAT CGT GGT GTC ACG CTC GTC GTT TGG TAT GGC TTC ATT CAG CTC

3350        3360        3370        3380        3390
 *           *           *           *           *
CGC TTC CCA ACG ATC AAG GCG AGT TAC ATG ATC CCC CAT GTT GTG CAA
```

FIG.23G

```
3400        3410        3420        3430        3440
 *           *           *           *           *
AAA AGC GGT TAG CTC CTT CGG TCC TCC GAT CGT TGT CAG AAG TAA GTT

 3450        3460        3470        3480        3490
  *           *           *           *           *
GGC CGC AGT GTT ATC ACT CAT GGT TAT GGC AGC ACT GCA TAA TTC TCT

  3500        3510        3520        3530        3540
   *           *           *           *           *
TAC TGT CAT GCC ATC CGT AAG ATG CTT TTC TGT GAC TGG TGA GTA CTC

    3550        3560        3570        3580
     *           *           *           *
AAC CAA GTC ATT CTG AGA ATA GTG TAT GCG GCG ACC GAG TTG CTC TTG

3590        3600        3610        3620        3630
 *           *           *           *           *
CCC GGC GTC AAC ACG GGA TAA TAC CGC GCC ACA TAG CAG AAC TTT AAA

3640        3650        3660        3670        3680
 *           *           *           *           *
AGT GCT CAT CAT TGG AAA ACG TTC TTC GGG CGG AAA ACT CTC AAG GAT

 3690        3700        3710        3720        3730
  *           *           *           *           *
CTT ACC GCT GTT GAG ATC CAG TTC GAT GTA ACC CAC TCG TGC ACC CAA

  3740        3750        3760        3770        3780
   *           *           *           *           *
CTG ATC TTC AGC ATC TTT TAC TTT CAC CAG CGT TTC TGG GTG AGC AAA

    3790        3800        3810        3820
     *           *           *           *
AAC AGG AAG GCA AAA TGC CGC AAA AAA GGG AAT AAG GGC GAC ACG GAA

3830        3840        3850        3860        3870        3880
 *           *           *           *           *           *
ATG TTG AAT ACT CAT ACTCTT CCTTTTTCAA TATTATTGAA GCATTTATCA
```

FIG.23H

```
      3890       3900       3910       3920       3930       3940
        *          *          *          *          *          *
GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG

      3950       3960       3970       3980       3990       4000
        *          *          *          *          *          *
GGTTCCGCGC ACATTTCCCC GAAAAGTGCC ACCTGACGTC TAAGAAACCA TTATTATCAT

      4010       4020       4030       4040       4050       4060
        *          *          *          *          *          *
GACATTAACC TATAAAAATA GGCGTATCAC GAGGCCCTTT CGTCTTCAAG AATTCTCATG
      pBR322#24  pBR322#340                          pBR322#375

      4070       4080       4090       4100       4110       4120
        *          *          *          *          *          *
TTTGACAGCTTAT CATCGAC TACGCGATCA TGGCCACCAC ACCCGTCCTGTG GATCTCTC
                                                                Lambda#35715

      4130       4140       4150       4160       4170       4180
        *          *          *          *          *          *
ACCTACCAAA CAATGCCCCC CTGCAAAAAA TAAATTCATA TAAAAAACAT ACAGATAACC

      4190       4200       4210       4220       4230       4240
        *          *          *          *          *          *
ATCTGCGGTG ATAAATTATC TCTGGCGGTG TTGACATAAA TACCACTGGC GGTGATACTG
pL transcriptional start

      4250       4260       4270       4280       4290       4300
        *          *          *          *          *          *
AGCACATCAG CAGGACGCAC TGACCACCAT GAAGGTGACG CTCTTAAAAA TTAAGCCCTG

      4310       4320       4330       4340       4350       4360
        *          *          *          *          *          *
AAGAAGGGCA GCATTCAAAG CAGAAGGCTT TGGGGTGTGT GATACGAAAC GAAGCATTGG

      4370       4380       4390       4400       4410       4420
        *          *          *          *          *          *
CCGTAAGTGC GATTCCGGAT TAGCTGCCAA TGTGCCAATC GCGGGGGGTT TTCGTTCAGG
```

FIG.23I

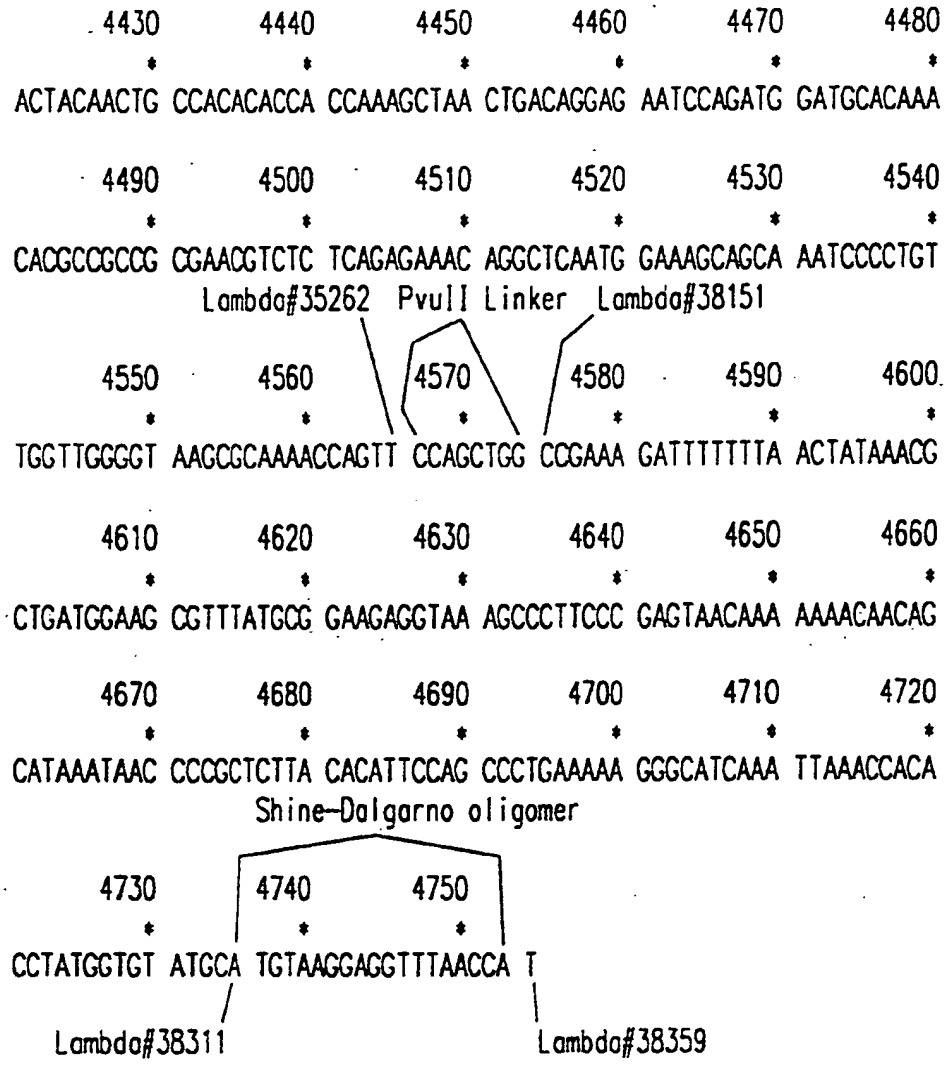
4430 4440 4450 4460 4470 4480
ACTACAACTG CCACACACCA CCAAAGCTAA CTGACAGGAG AATCCAGATG GATGCACAAA
4490 4500 4510 4520 4530 4540
CACGCCGCCG CGAACGTCTC TCAGAGAAAC AGGCTCAATG GAAAGCAGCA AATCCCCTGT
Lambda#35262 PvuII Linker Lambda#38151
4550 4560 4570 4580 4590 4600
TGGTTGGGGT AAGCGCAAAACCAGTT CCAGCTGG CCGAAA GATTTTTTTA ACTATAAACG
4610 4620 4630 4640 4650 4660
CTGATGGAAG CGTTTATGCG GAAGAGGTAA AGCCCTTCCC GAGTAACAAA AAAACAACAG
4670 4680 4690 4700 4710 4720
CATAAATAAC CCCGCTCTTA CACATTCCAG CCCTGAAAAA GGGCATCAAA TTAAACCACA
Shine-Dalgarno oligomer
4730 4740 4750
CCTATGGTGT ATGCA TGTAAGGAGGTTTAACCA T
Lambda#38311
Lambda#38359

FIG.23J

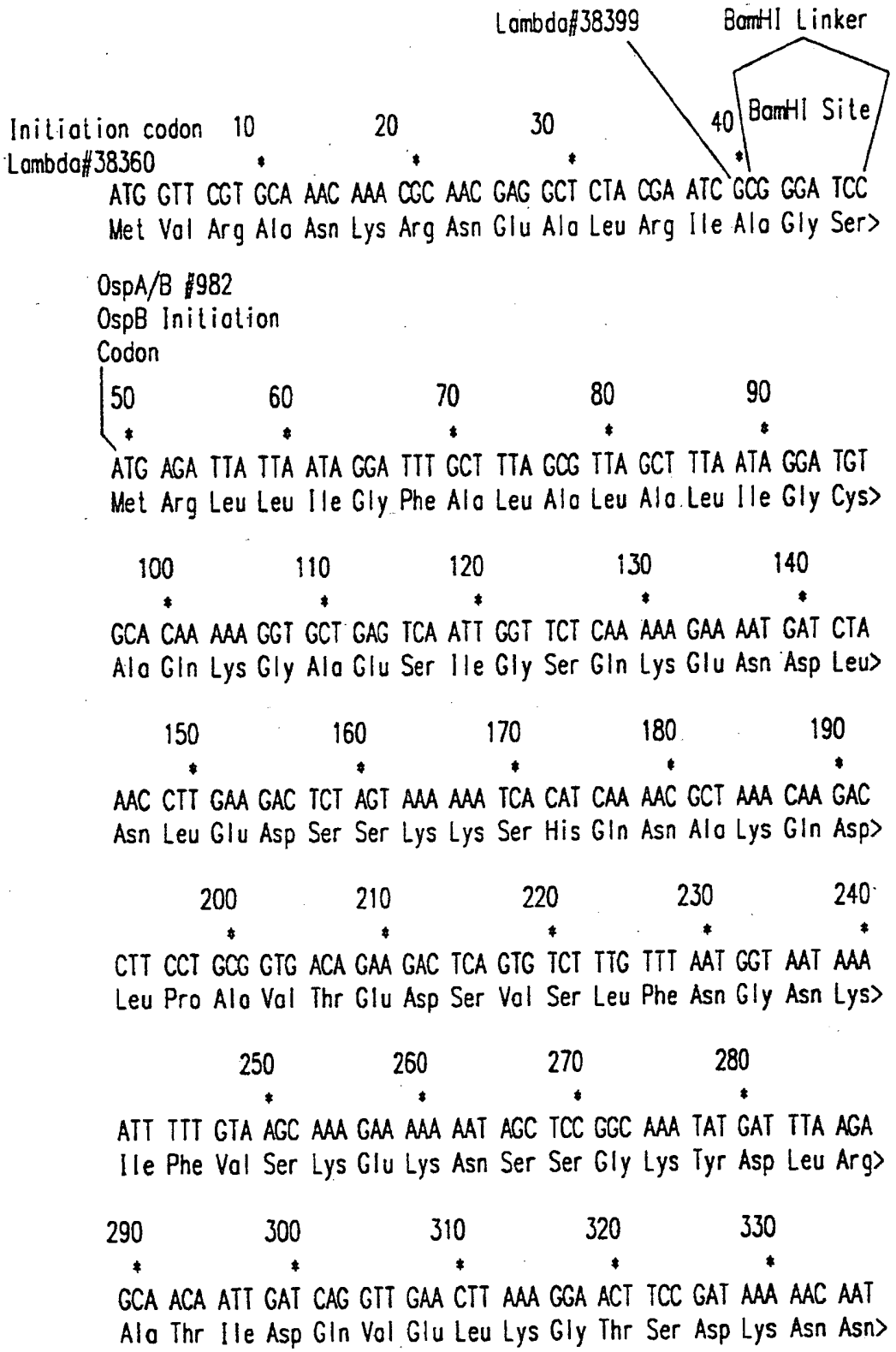
Lambda#38399
BamHI Linker
BamHI Site
Initiation codon
Lambda#38360
10
20
30
40
ATG GTT CGT GCA AAC AAA CGC AAC GAG GCT CTA CGA ATC GCG GGA TCC
Met Val Arg Ala Asn Lys Arg Asn Glu Ala Leu Arg Ile Ala Gly Ser>
OspA/B #982
OspB Initiation
Codon
50
60
70
80
90
ATG AGA TTA TTA ATA GGA TTT GCT TTA GCG TTA GCT TTA ATA GGA TGT
Met Arg Leu Leu Ile Gly Phe Ala Leu Ala Leu Ala Leu Ile Gly Cys>
100
110
120
130
140
GCA CAA AAA GGT GCT GAG TCA ATT GGT TCT CAA AAA GAA AAT GAT CTA
Ala Gln Lys Gly Ala Glu Ser Ile Gly Ser Gln Lys Glu Asn Asp Leu>
150
160
170
180
190
AAC CTT GAA GAC TCT AGT AAA AAA TCA CAT CAA AAC GCT AAA CAA GAC
Asn Leu Glu Asp Ser Ser Lys Lys Ser His Gln Asn Ala Lys Gln Asp>
200
210
220
230
240
CTT CCT GCG GTG ACA GAA GAC TCA GTG TCT TTG TTT AAT GGT AAT AAA
Leu Pro Ala Val Thr Glu Asp Ser Val Ser Leu Phe Asn Gly Asn Lys>
250
260
270
280
ATT TTT GTA AGC AAA GAA AAA AAT AGC TCC GGC AAA TAT GAT TTA AGA
Ile Phe Val Ser Lys Glu Lys Asn Ser Ser Gly Lys Tyr Asp Leu Arg>
290
300
310
320
330
GCA ACA ATT GAT CAG GTT GAA CTT AAA GGA ACT TCC GAT AAA AAC AAT
Ala Thr Ile Asp Gln Val Glu Leu Lys Gly Thr Ser Asp Lys Asn Asn>

FIG.24A

```
  340         350         360         370         380
   *           *           *           *           *
GGT TCT GGA ACC CTT GAA GGT TCA AAG CCT GAC AAG AGT AAA GTA AAA
Gly Ser Gly Thr Leu Glu Gly Ser Lys Pro Asp Lys Ser Lys Val Lys>

    390         400         410         420         430
     *           *           *           *           *
TTA ACA GTT TCT GCT GAT TTA AAC ACA GTA ACC TTA GAA GCA TTT GAT
Leu Thr Val Ser Ala Asp Leu Asn Thr Val Thr Leu Glu Ala Phe Asp>

       440         450         460         470         480
        *           *           *           *           *
GCC AGC AAC CAA AAA ATT TCA AGT AAA GTT ACT AAA AAA CAG GGG TCA
Ala Ser Asn Gln Lys Ile Ser Ser Lys Val Thr Lys Lys Gln Gly Ser>

          490         500         510         520
           *           *           *           *
ATA ACA GAG GAA ACT CTC AAA GCT AAT AAA TTA GAC TCA AAG AAA TTA
Ile Thr Glu Glu Thr Leu Lys Ala Asn Lys Leu Asp Ser Lys Lys Leu>

530         540         550         560         570
 *           *           *           *           *
ACA AGA TCA AAC GGA ACT ACA CTT GAA TAC TCA CAA ATA ACA GAT GCT
Thr Arg Ser Asn Gly Thr Thr Leu Glu Tyr Ser Gln Ile Thr Asp Ala>

  580         590         600         610         620
   *           *           *           *           *
GAC AAT GCT ACA AAA GCA GTA GAA ACT CTA AAA AAT AGC ATT AAG CTT
Asp Asn Ala Thr Lys Ala Val Glu Thr Leu Lys Asn Ser Ile Lys Leu>

    630         640         650         660         670
     *           *           *           *           *
GAA GGA AGT CTT GTA GTC GGA AAA ACA ACA GTG GAA ATT AAA GAA GGT
Glu Gly Ser Leu Val Val Gly Lys Thr Thr Val Glu Ile Lys Glu Gly>

       680         690         700         710         720
        *           *           *           *           *
ACT GTT ACT CTA AAA AGA GAA ATT GAA AAA GAT GGA AAA GTA AAA GTC
Thr Val Thr Leu Lys Arg Glu Ile Glu Lys Asp Gly Lys Val Lys Val>

          730         740         750         760
           *           *           *           *
TTT TTG AAT GAC ACT GCA GGT TCT AAC AAA AAA ACA GGT AAA TGG GAA
Phe Leu Asn Asp Thr Ala Gly Ser Asn Lys Lys Thr Gly Lys Trp Glu>
```

FIG.24B

```
770         780         790         800         810
 *           *           *           *           *
GAC AGT ACT AGC ACT TTA ACA ATT AGT GCT GAC AGC AAA AAA ACT AAA
Asp Ser Thr Ser Thr Leu Thr Ile Ser Ala Asp Ser Lys Lys Thr Lys>

 820         830         840         850         860
  *           *           *           *           *
GAT TTG GTG TTC TTA ACA GAT GGT ACA ATT ACA GTA CAA CAA TAC AAC
Asp Leu Val Phe Leu Thr Asp Gly Thr Ile Thr Val Gln Gln Tyr Asn>

  870         880         890         900         910
   *           *           *           *           *
ACA GCT GGA ACC AGC CTA GAA GGA TCA GCA AGT GAA ATT AAA AAT CTT
Thr Ala Gly Thr Ser Leu Glu Gly Ser Ala Ser Glu Ile Lys Asn Leu>
                                  POLY-TRANSLATION TERMINATION OLIGOMER
      920         930         940 |       950         960
       *           *           * |        *           *
TCA GAG CTT AAA AAC GCT TTA AAA TAAT ATGGATCCTA GGTAAGTAGG
Ser Glu Leu Lys Asn Ala Leu Lys>  |    \OspA/B #1875
                                  |
pBR322#652                        Stop codon OspB
|     970         980         990        1000        1010        1020
|      *           *           *          *           *           *
TCGACCGATG CCCTTGAGAG CCTTCAACCC AGTCAGCTCC TTCCGGTGGG CGCGGGGCAT

     1030        1040        1050        1060        1070        1080
      *           *           *           *           *           *
GACTATCGTC GCCGCACTTA TGACTGTCTT CTTTATCATG CAACTCGTAG GACAGGTGCC

     1090        1100        1110        1120        1130        1140
      *           *           *           *           *           *
GGCAGCGCTC TGGGTCATTT TCGGCGAGGA CCGCTTTCGC TGGAGCGCGA CGATGATCGG

     1150        1160        1170        1180        1190        1200
      *           *           *           *           *           *
CCTGTCGCTT GCGGTATTCG GAATCTTGCA CGCCCTCGCT CAAGCCTTCG TCACTGGTCC

     1210        1220        1230        1240        1250        1260
      *           *           *           *           *           *
CGCCACCAAA CGTTTCGGCG AGAAGCAGGC CATTATCGCC GGCATGGCGG CCGACGCGCT
```

FIG.24C

```
1270        1280        1290        1300        1310        1320
*           *           *           *           *           *
GGGCTACGTC TTGCTGGCGT TCGCGACGCG AGGCTGGATG GCCTTCCCCA TTATGATTCT

1330        1340        1350        1360        1370        1380
*           *           *           *           *           *
TCTCGCTTCC GGCGGCATCG GGATGCCCGC GTTGCAGGCC ATGCTGTCCA GGCAGGTAGA

1390        1400        1410        1420        1430        1440
*           *           *           *           *           *
TGACGACCAT CAGGGACAGC TTCAAGGATC GCTCGCGGCT CTTACCAGCC TAACTTCGAT

1450        1460        1470        1480        1490        1500
*           *           *           *           *           *
CACTGGACCG CTGATCGTCA CGGCGATTTA TGCCGCCTCG GCGAGCACAT GGAACGGGTT

1510        1520        1530        1540        1550        1560
*           *           *           *           *           *
GGCATGGATT GTAGGCGCCG CCCTATACCT TGTCTGCCTC CCCGCGTTGC GTCGCGGTGC

1570        1580        1590        1600        1610        1620
*           *           *           *           *           *
ATGGAGCCGG GCCACCTCGA CCTGAATGGA AGCCGGCGGC ACCTCGCTAA CGGATTCACC

1630        1640        1650        1660        1670        1680
*           *           *           *           *           *
ACTCCAAGAA TTGGAGCCAA TCAATTCTTG CGGAGAACTG TGAATGCGCA AACCAACCCT

1690        1700        1710        1720        1730        1740
*           *           *           *           *           *
TGGCAGAACA TATCCATCGC GTCCGCCATC TCCAGCAGCC GCACGCGGCG CATCTCGGGC
    pBR322#1446  pBR322#2069

1750        1760        1770        1780        1790        1800
*           *           *           *           *           *
AGCGTTGGGT CCTGGCTGCC TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT

1810        1820        1830        1840        1850        1860
*           *           *           *           *           *
GCAGCTCCCG GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG
```

FIG.24D

```
1870        1880        1890        1900        1910        1920
*           *           *           *           *           *
TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCGCA GCCATGACCC AGTCACGTAG

1930        1940        1950        1960        1970        1980
*           *           *           *           *           *
CGATAGCGGA GTGTATACTG GCTTAACTAT GCGGCATCAG AGCAGATTGT ACTGAGAGTG

1990        2000        2010        2020        2030        2040
*           *           *           *           *           *
CACCATATGC GGTGTGAAAT ACCGCACAGA TGCGTAAGGA GAAAATACCG CATCAGGCGC

2050        2060        2070        2080        2090        2100
*           *           *           *           *           *
TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA

2110        2120        2130        2140        2150        2160
*           *           *           *           *           *
TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG

2170        2180        2190        2200        2210        2220
*           *           *           *           *           *
AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG

2230        2240        2250        2260        2270        2280
*           *           *           *           *           *
TTTTTCCATA GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG

2290        2300        2310        2320        2330        2340
*           *           *           *           *           *
TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG

2350        2360        2370        2380        2390        2400
*           *           *           *           *           *
CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA

2410        2420        2430        2440        2450        2460
*           *           *           *           *           *
AGCGTGGCGC TTTCTCAATG CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC
```

FIG.24E

```
2470        2480        2490        2500        2510        2520
 *           *           *           *           *           *
TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT

2530        2540        2550        2560        2570        2580
 *           *           *           *           *           *
AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT

2590        2600        2610        2620        2630        2640
 *           *           *           *           *           *
GGTAACAGGA TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG

2650        2660        2670        2680        2690        2700
 *           *           *           *           *           *
CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT

2710        2720        2730        2740        2750        2760
 *           *           *           *           *           *
ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT

2770        2780        2790        2800        2810        2820
 *           *           *           *           *           *
GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT

2830        2840        2850        2860        2870        2880
 *           *           *           *           *           *
TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG

2890        2900        2910        2920        2930        2940
 *           *           *           *           *           *
GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT

2950        2960        2970        2980        2990        3000
 *           *           *           *           *           *
AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT

3010        3020        3030        3040        3050        3060
 *           *           *           *           *           *
GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG ACTCCCCGTC
```

FIG.24F

```
3070        3080        3090        3100        3110        3120
*           *           *           *           *           *
GTGTAGATAA CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG

3130        3140        3150        3160        3170        3180
*           *           *           *           *           *
CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC

3190        3200        3210        3220        3230        3240
*           *           *           *           *           *
GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG

3250        3260        3270        3280        3290        3300
*           *           *           *           *           *
GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTGCA

3310        3320        3330        3340        3350        3360
*           *           *           *           *           *
GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA

3370        3380        3390        3400        3410        3420
*           *           *           *           *           *
TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT

3430        3440        3450        3460        3470        3480
*           *           *           *           *           *
CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG

3490        3500        3510        3520        3530        3540
*           *           *           *           *           *
CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA

3550        3560        3570        3580        3590        3600
*           *           *           *           *           *
ACCAAGTCAT TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC GGCGTCAACA

3610        3620        3630        3640        3650        3660
*           *           *           *           *           *
CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT

3670        3680        3690        3700        3710        3720
*           *           *           *           *           *
TCGGGGCGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT
```

FIG.24G

```
      3730        3740        3750        3760        3770        3780
       *           *           *           *           *           *
CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA

      3790        3800        3810        3820        3830        3840
       *           *           *           *           *           *
ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC

      3850        3860        3870        3880        3890        3900
       *           *           *           *           *           *
ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA

      3910        3920        3930        3940        3950        3960
       *           *           *           *           *           *
TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA

      3970        3980        3990        4000        4010        4020
       *           *           *           *           *           *
AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG
                                                  pBR322#24 pBR322#340
      4030        4040        4050        4060        4070        4080
       *           *           *           *           *           *
CGTATCACGA GGCCCTTTCG TCTTCAAGAA TTCTCATGTT TGACAGCTTA TCATCGACTA
                          pBR322#375  Lambda#35715
      4090        4100        4110        4120        4130        4140
       *           *           *           *           *           *
CGCGATCATG GCCACCACAC CCGTCCTGTG GATCTCTCAC CTACCAAACA ATGCCCCCCT

      4150        4160        4170        4180        4190        4200
       *           *           *           *           *           *
GCAAAAAATA AATTCATATA AAAAACATAC AGATAACCAT CTGCGGTGAT AAATTATCTC

      4210        4220        4230        4240        4250        4260
       *           *           *           *           *           *
TGGCGGTGTT GACATAAATA CCACTGGCGG TGATACTGAG CACATCAGCA GGACGCACTG
                                                 pL trascriptional start
      4270        4280        4290        4300        4310        4320
       *           *           *           *           *           *
ACCACCATGA AGGTGACGCT CTTAAAAATT AAGCCCTGAA GAAGGGCAGC ATTCAAAGCA
```

FIG.24H

```
      4330        4340        4350        4360        4370        4380
       *           *           *           *           *           *
GAAGGCTTTG GGGTGTGTGA TACGAAACGA AGCATTGGCC GTAAGTGCGA TTCCGGATTA

      4390        4400        4410        4420        4430        4440
       *           *           *           *           *           *
GCTGCCAATG TGCCAATCGC GGGGGGTTTT CGTTCAGGAC TACAACTGCC ACACACCACC

      4450        4460        4470        4480        4490        4500
       *           *           *           *           *           *
AAAGCTAACT GACAGGAGAA TCCAGATGGA TGCACAAACA CGCCGCCGCG AACGTCTCTC

      4510        4520        4530        4540        4550        4560
       *           *           *           *           *           *
AGAGAAACAG GCTCAATGGA AAGCAGCAAA TCCCCTGTTG GTTGGGGTAA GCGCAAAACC
Lambda#35262  PvuII Linker  Lambda#38151
      4570        4580        4590        4600        4610        4620
       *           *           *           *           *           *
AGTT CCAGCTGG CCGAAAGA TTTTTTTAAC TATAAACGCT GATGGAAGCG TTTATGCGGA

      4630        4640        4650        4660        4670        4680
       *           *           *           *           *           *
AGAGGTAAAG CCCTTCCCGA GTAACAAAAA AACAACAGCA TAAATAACCC CGCTCTTACA
                                                 Lambda#38311
      4690        4700        4710        4720        4730        4740
       *           *           *           *           *           *
CATTCCAGCC CTGAAAAAGG GCATCAAATT AAACCACACC TATGGTGTATGCA TGTAAGG
                                                          |
                                                  Shine-Dalgarno

AGGTTTAACCA T
Oligomer    Lambda#38359
```

FIG.24I

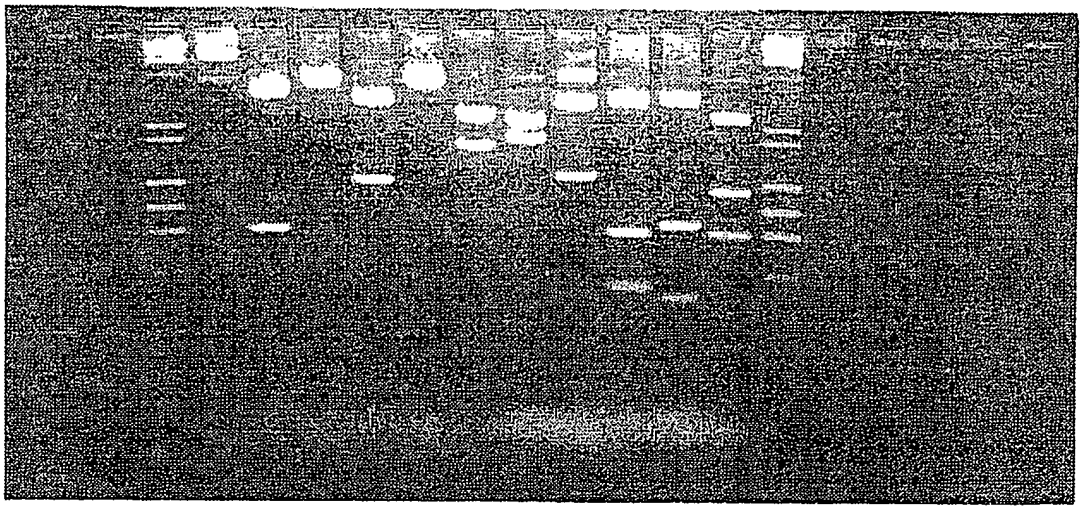

FIG. 25

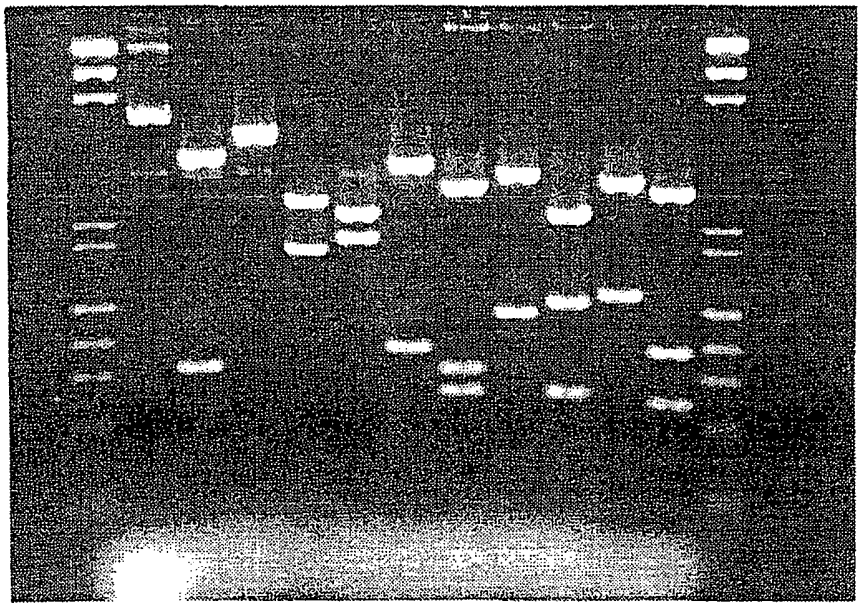

FIG. 26

INTERNATIONAL SEARCH REPORT

In: tional application No.
PCT/US94/02095

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :G01N 33/569; A61K 39/02
US CL :435/7.32, 29; 424/7.1, 92
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.32, 4, 29, 967; 424/7.1, 92

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Journal of Microbiological Methods, Volume 17, Number 2, issued March 1993, Ma et al, "A simple, colorimetric microtiter assay for borreliacidal activity of antisera", pages 145-153, see paragraph bridging pages 146-147.	1-4
Y	Journal of Infectious Diseases, Volume 163, issued March 1991, Pavia et al, "Antiborrelial Activity of Serum from Rats Injected wtih the Lyme Disease Spirochete", pages 656-659, see "Inhibition of borrelial growth" on page 657.	1-4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
28 APRIL 1994

Date of mailing of the international search report
19 MAY 1994

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02095

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Clinical Microbiology, Volume 30, Number 10, issued October 1992, Dever et al, "In Vitro Antimicrobial Susceptibility Testing of Borrelia burgdorferi: a Microdilution MIC Method and Time-Kill Studies", pages 2692-2697, see paragraph bridging pages 2692-2693.	1-4
Y	The Yale Journal of Biology and Medicine, Volume 57, Number 4, issued August 1984, Barbour, "Isolation and Cultivation of Lyme Disease Spirochetes", pages 521-525, see Table 1 on page 523.	1-4
A	Journal of Clinical Microbiology, Volume 7, Number 1, issued January 1978, Murphy et al, "Determination of Corynebacterium diphtheriae Toxigenicity by a Colorimetric Tissue Culture Assay", pages 91-96.	1-4
Y	1990 GIBCO BRL Catalogue Reference Guide, published 1990 by Life Technologies, Inc., (Gaithersburg, MD), pages 94-95, see entire document.	1-4
Y	Difco Manual, Dehydrated Culture Media and Reagents for Microbiology, 10th Edition, published 1984 by Difco Laboratories, Inc., (Detroit, MI), pages 660-662, see page 661, first paragraph.	1-4
Y	US, A, 4,806,350 (GERBER) 21 February 1989, see entire document, especially Col. 2, lines 14-28.	5-23
Y	US, A, 4,721,617 (JOHNSON) 26 January 1988, see entire document, especially see col. 2, lines 8-16.	5-23
Y	International Journal of Systemic Bacteriology, Volume 42, Number 3, issued July 1992, Baranton et al, "Delineation of Borrelia burgdorferi Sensu Stricto, Borrelia garinii sp. nov., and Group VS461 Associated with Lyme Borreliosis", pages 378-383, see Abstract on page 378.	5-23
Y	Infection and Immunity, Volume 60, Number 2, issued February 1992, Fikrig et al, "Roles of OspA, OspB, and Flagellin in Protective Immunity to Lyme Borreliosis in Laboratory Mice", pages 657-661, see Abstract on page 657 and page 658 paragraph bridging cols. 1-2.	5-23

INTERNATIONAL SEARCH REPORT

Int ..tional application No.
PCT/US94/02095

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Immunology, Volume 148, Number 7, issued 01 April 1992, Fikrig et al, "Borrelia burgdorferi Strain 25015: Characterization of Outer Surface Protein A and Vaccination Against Infection", pages 2256-2260, see Abstract on page 2256.	5-23
Y	Infection and Immunity, Volume 61, Number 1, issued January 1993, Erdile et al, "Role of Attached Lipid in Immunogenicity of Borrelia burgdorferi OspA", pages 81-90, see page 81, third paragraph.	5-23
Y	Journal of Immunology, Volume 146, Number 2, issued 15 January 1991, Kensil et al, "Separation and Characterization of Saponins with adjuvant activity from Quillaja saponaria Molina Cortex", pages 431-437, ee page 433, col. 2, second complete paragraph.	5-23
A	Vaccine, Volume 9, Number 2, issued 1991, Marciani et al, "Genetically-engineered subunit vaccine against feline leukaemia virus: protective immune response in cats" pages 89-96.	5-23

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/US94/02095

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Telephone Practice
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-4, drawn to methods using antiserum against Borrelia burgdorferi to kill or inhibit B. burgdorferi or to detect Lyme borreliosis.

II. Claims 5-23, drawn to vaccine comprising OspA, OspB or fragments thereof and a saponin adjuvant and a method of inducing immunity by administering the vaccine.

The methods of Group I do not use or require the vaccine of Group II. The methods of Groups I and II use different materials in different method steps to achieve different end results. The inventions of Group I and II do not share a special technical feature so as to form a single general inventive concept and thus do not meet the criteria set forth in PCT Rules 13.1 ot 13.3.